IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Appellant:		§	
		§	
	Luba COHEN, et al	§	
		§	
Serial No.:	09/955,933	§	
		§	
Filed:	September 20, 2001	§	Group Art Unit: 1651
		§	
For:	LICORICE EXTRACT FOR USE AS A MEDICAMENT	§	
		§	Attorney Docket: **37229**
Examiner:	Ware, Deborah K	§	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPEAL BRIEF

Sir:

This brief is further to a notice of appeal filed July 17, 2008 to the final rejection issued on April 18, 2008.

Please charge the Appeal Brief fee of $270 to Deposit Account 50-1407.

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REAL PARTY OF INTEREST

The real party in interest of this appeal is F&C Licorice Ltd. of which the assignee Ready-Made 37 Ltd. is a fully owned subsidiary.

RELATED APPEALS AND INTERFERENCES

This appeal has no related proceedings or interferences.

STATUS OF CLAIMS

A. TOTAL NUMBER OF CLAIMS IN THE APPLICATION

The claims in the application are: 1-33.

B. STATUS OF ALL THE CLAIMS IN THE APPLICATION

Claims canceled: 3, 4, 6, 12 and 18.

Claims withdrawn from consideration but not cancelled: NONE

Claims pending: 1, 2, 5, 7-11, 13-17, 19-33.

Claims allowed: NONE

Claims rejected: 1, 2, 5, 7-11, 13-17, 19-33.

Claims objected to: NONE

C. CLAIMS ON APPEAL

The claims on appeal are: 1, 2, 5, 7-11, 13-17, 19-33.

STATUS OF AMENDMENTS

An amendment after final rejection was not filed. Therefore, claims 1, 2, 5, 7-11, 13-17, 19-33 on appeal are as amended in the response to office action filed January 8, 2008.

SUMMARY OF CLAIMED SUBJECT MATTER

The independent claims in the application are claims 1, 9, 10, 23 and 30. Appellants will separately argue dependent claims 1, 2, 5, 8-10, 23-26 and 28-32. The argued claims are repeated below with reference to passages in the application as published providing support, in bold letters.

Independent claim 1 claims a method for lowering at least one risk factor in a patient suffering from said risk factor, comprising

identifying that said patient suffers from said at least one risk factor; and **{original claims and inherent in a method of lowering a risk factor}**

administering to said patient an effective amount of a licorice extract which is water-insoluble and free from glycyrrhizinic acid, thereby lowering said risk factor, wherein said at least one risk factor comprises a risk factor selected from the group consisting of high blood pressure, high blood glucose concentration, and high blood triglycerides concentration.**{paragraphs 0006, 0011 and 0012}**

Claim 2, dependent on claim 1, limits the scope of claim 1 so that one of said at least one risk factor is high blood pressure. **{paragraphs 0006, 0011 and 0012}**

Claim 5, dependent on claim 1, limits the scope of claim 1 so that one of said at least one risk factor is high blood glucose concentration. **{paragraphs 0006, 0011 and 0012}**

Claim 26, dependent on claim 1, limits the scope of claim 1 so that one of said at least one risk factor is high blood triglycerides concentration.

Claim 7, dependent on claim 1, limits the scope of claim 1 so that said patient suffers from at least two risk factors, at least one of which is selected from the group consisting of high blood pressure, high blood glucose concentration, and high blood triglycerides concentration (these are the factors listed in claim 1), and at least one selected from the group consisting of high LDL (low-density lipoprotein) susceptibility to retention, high LDL susceptibility to aggregation, high blood total cholesterol, high

LDL levels, and high VLDL (very low-density lipoprotein) concentration. **{paragraphs 0006, 0011 and 0012}**

Claim 8, dependent on claim 7, limits the scope of claim 7 so that the method for treating a patient suffering from high blood triglycerides and high LDL (low-density lipoprotein) levels without decreasing the HDL (high density lipoprotein) level of said patient, comprising **{paragraph 0034}**

administering to said patient an effective amount of a licorice extract which is water-insoluble and free from glycyrrhizinic acid, thereby treating the high blood triglycerides and high LDL levels without decreasing the HDL level of said patient. **{paragraphs 0006 and 0034}**

Claim 33, dependent on claim 1, limits the scope of claim 1 so that administering to said patient an effective amount of a licorice extract consists of administering to said patient a pharmaceutical or nutraceutical preparation, said preparation being free from water-soluble licorice extract. **{paragraphs 0001 and 0029}**

Independent claim 9 claims a method for treating a patient suffering from at least one condition, comprising

identifying that said patient suffers from said at least one condition; and **{original claims and inherent in a method of treatment}**

administering to said patient an effective amount of a licorice extract which is water-insoluble and free from glycyrrhizinic acid, wherein said at least one condition is selected from the group consisting of hypertension and chronic renal failure. **{paragraphs 0009, 0011 and 0012}**

Claim 28, dependent on claim 9, limits the scope of claim 9 so that one of said at least one condition is hypertension. **{paragraphs 0009, 0011 and 0012}**

Claim 29, dependent on claim 9, limits the scope of claim 9 so that one of said at least one condition is chronic renal failure. **{paragraphs 0009, 0011 and 0012}**

Independent claim 10 claims a method for preventing a patient from suffering from at least one condition, comprising

identifying that said patient is in high risk to suffer from said at least one condition; and **{original claims and inherent in a method of preventing a condition}**

administering to said patient an effective amount of a licorice extract which is water-insoluble and free from glycyrrhizinic acid, wherein said at least one condition is selected from the group consisting of hypertension and chronic renal failure. **{paragraphs 0009, 0011 and 0012}**

Independent claim 23 claims a method for lowering at least one risk factor in a patient suffering from said at least one risk factor, comprising

identifying that said patient suffers from said at least one risk factor; and **{original claims and inherent in a method of lowering risk factors}**

administering to said patient an effective amount of a licorice extract which is water-insoluble and free from glycyrrhizinic acid, thereby lowering said risk factor, wherein said at least one risk factor comprises a risk factor selected from the group consisting of high blood total cholesterol, high blood LDL levels, high blood triglycerides concentration and high blood VLDL (very low-density lipoprotein) concentration. **{paragraphs 0006, 0011 and 0012}**

Claim 24, dependent on claim 23, limits the scope of claim 23 so that one of said at least one risk factor is high blood total cholesterol. **{paragraphs 0006, 0011 and 0012}**

Claim 25, dependent on claim 23, limits the scope of claim 23 so that one of said at least one risk factor is high LDL levels. **{paragraphs 0006, 0011 and 0012}**

Claim 27, dependent on claim 23, limits the scope of claim 23 so that one of said at least one risk factor is high blood VLDL concentration. **{paragraphs 0006, 0011 and 0012}**

Independent claim 30 claims a method for treating a patient suffering from at least one condition, comprising

identifying that said patient suffers from said at least one condition; and **{original claims and inherent in a method of treatment}**

administering to said patient an effective amount of a licorice extract which is water-insoluble and free from glycyrrhizinic acid, wherein said at least one condition comprises a condition selected from the group consisting of hypercholestoremia and hypertriglyceridemia. **{paragraphs 0009, 0011 and 0012}**

Claim 31, dependent on claim 30, limits the scope of claim 30 so that one of said at least one condition is hypercholesterolemia.

Claim 32, dependent on claim 30, limits the scope of claim, 30 so that one of said at least one condition is hypertriglyceridemia.

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

There is only one ground of rejection pending and to be reviewed on appeal:

Claims 1, 2, 5, 7-11, 13-17 and 19-33 stand rejected under 35 USC 103(a) as being unpatentable over Sha et al (US 6,280,776) in view of Fuhrman et al (The American Journal of Clinical Nutrition).

ARGUMENTS

Note: All prior art references referred to below have been previously presented to the Examiner or cited by the Examiner.

Claims 1, 2, 5, 7-11, 13-17 and 19-33 stand rejected under 35 USC 103(a) as being unpatentable over Sha et al (US 6,280,776) in view of Fuhrman et al (The American Journal of Clinical Nutrition).

Claims 1, 9, 10, 23 and 30 are the only independent claims in this group of claims.

Fuhrman teaches the use of a licorice extract which is water ***insoluble*** and ***free from*** glycyrrhizinic acid for reducing LDL susceptibility to oxidation and reducing atherosclerotic lesion in artherosclerotic mice.

Sha describes the use of a different extract, namely a water ***soluble*** licorice extract ***containing*** glycyrrhizinic acid for treating risk factors or conditions, such as high blood sugar.

The claims in the present application relate to the use of a licorice extract which is water insoluble and free from glycyrrhizinic acid, similarly to the extract disclosed by Fuhrman, for treating, preventing or lowering certain risk factors and conditions, some of which are similar to *some* of those treated by Sha. Fuhrman does not teach treating any of the claimed conditions.

The Examiner states that a person of ordinary skill in the art would replace the licorice extract disclosed by Sha with that disclosed by Fuhrman and would thereby achieve the claimed invention. The reasoning provided by the Examiner is that one of skill would have been motivated to minimize LDL oxidation in Sha to achieve enhanced expected results of lowering the risk factors and conditions of these problematic diseases associated with LDL oxidation (page 3, paragraph 4 of the final office action).

Appellants respectfully disagree with the rejection and submit that the Examiner has not provided a *prima facie* case of obviousness for a number of reasons.

Firstly, the extracts of Sha and Fuhrman are completely different chemically, see paragraphs 12-16 of the Vaya declaration. While the motivation supplied by the Examiner might be sufficient to motivate a person of the art to replace a particular compound by another compound (if indeed the motivation were correct, which it is not), it would hardly suffice to motivate one to replace one compound which was found to be

effective by another which was not found to be effective.

Secondly, there is no explicit teaching or indication in either reference to utilize the claimed extract to treat any of the risk factors claimed. Fuhrman does not teach using her extract to treat risk factors as claimed, and in fact teaches against using her extract to treat some of the claimed risk factors. Sha teaches against using an extract such as that used by Fuhrman for treating the claimed risk factors and conditions, let alone replacing his extract by Fuhrman's extract. It is difficult to see how one could be motivated to treat or prevent the risk factors of Sha utilizing a different material which is not taught as being effective for these uses. This lack of teaching goes well beyond a "lack of *teaching* of motivation." There is just no reason, based on the references cited to utilize the extract of Fuhrman for any of the conditions claimed.

Thirdly, there is no motivation for combining the references. Any such combination is at odds with what was known in the art at the time of the invention. In this regard, appellants submitted affidavits by Michael AVIRAM and Prof. Jacob VAYA, both co-authors of Fuhrman to demonstrate what a person of the art was aware of at the time of the invention. It is clear from both affidavits that a person of ordinary skill in the art, at the time of the invention, would not find it obvious to combine the references as suggested by the Examiner. Moreover, as stated in paragraph 19 of the Vaya affidavit, the combination suggested by the Examiner is against common sense. A copy of the affidavits are attached hereto and marked Annexes A and B. The discussion below references where in each affidavit these statements of the art are listed.

Fourthly, Fuhrman is not only silent about some of the risk factors, but actually specifically teaches against using his extract for some of the conditions claimed.

The claims which are seperately argued claim treatment, lowering or prevention of the following risk factors:

Independent claim 1 claims lowering of one or more of the following risk factors:

high blood pressure, high blood glucose concentration, and high blood triglycerides concentration.

Claim 2 depends on claim 1 and specifically claims lowering high blood pressure.

Claim 5 depends on claim 1 and specifically claims lowering high blood glucose

concentration.

Claim 8 depends indirectly on claim 1 and specifically claims treating the high blood triglycerides and high LDL levels without decreasing the HDL level of the patient.

Claim 26 depends on claim 1 and specifically claims lowering high blood triglycerides concentration.

Independent claim 9 claims treatment of one or more of the following conditions:

hypertension and chronic renal failure.

Claim 28 depends on claim 9 and claims specifically treatment of hypertension.

Claim 29 depends on claim 9 and claims specifically treatment of chronic renal failure.

Independent claim 10 claims *preventing* of one or both of the following conditions:

hypertension and chronic renal failure.

Independent claim 23 claims lowering of one or more of the following risk factors:

high blood total cholesterol, high blood LDL levels, high blood triglycerides concentration and high blood VLDL (very low-density lipoprotein) concentration.

Claim 24 depends on claim 23 and specifically claims lowering high blood total cholesterol.

Claim 25 depends on claim 23 and specifically claims lowering high LDL levels.

Independent claim 30 claims *treatment* of one or more of hypercholestoremia and hypertriglyceridemia.

Claim 31 depends on claim 30 and specifically claims treatment of hypercholestoremia.

Claim 32 depends on claim 30 and specifically claims treatment of hypertriglyceridemia.

Appellants submit that if treatment or prevention (as claimed) of none of the

factors listed above for any of claims 1, 9, 10, 23 or 30 are obvious using the extract claims than those claims and all their dependent claims are patentable. Even if a particular independent claim is unpatentable because of one of the factors, a dependent claim specifically claiming a single factor whose treatment/prevention is not found to be obvious would be patentable.

A. Fuhrman does not teach using her extract to treat risk factors as claimed, and in fact teaches against using her extract to treat some of the claimed risk factors.

Fuhrman describes the licorice extract reducing LDL susceptibility to oxidation, and reducing atherosclerotic lesion in atherosclerotic mice. However, there is no mention in Fuhrman to use the same extract for treating the risk factors claimed in any of the claims. On the contrary, Fuhrman teaches against using the same extract for some of the risk factors claimed. For example, Furman's data shows that various lipid levels in the blood were substantially unaffected by the extract (Results on page 270 of Fuhrman).

Not only is Furman silent about or teaches away from treating the risk factors with licorice extract, a person of skill in the art would not have expected that the extract would alleviate the other risk factors. This is supported by the Aviram declaration, paragraphs 5-9. The claimed risk factors and conditions are conventionally treated with medicaments different than those used to treat atherosclerosis. For instance, blood pressure is treated with one drug, and atherosclerosis with another. If a patient suffers from both, the patient is generally administered two different medications, one for each condition. Similarly, high cholesterol is conventionally treated with Statins which are unrelated to licorice extract. There would be no reasonable expectation of success in such treatment. Appellants submit that it would not rise to the level of "obvious to try" since obvious to try requires a reasonable expectation of success.

Appellants have raised these arguments in a previous response and the Examiner responded by quoting from the KSR decision. Appellants respectfully quote the following from Esaico Co v. Teva Pharms, 2007-1397:

"... In KSR, the Supreme Court noted that an invention may have been obvious "[w]hen there [was] . . . a design need or market pressure to solve a problem and there [were] . . . a finite number of identified, predictable solutions." 127 S. Ct. at 1742 (tense changes supplied to clarify, as the Court stated and as per 35 U.S.C. § 103, that the obviousness inquiry must rely on evidence available "at the time" of the invention, see Takeda, 492 F.3d at 1356 n.2). The Supreme Court's analysis in KSR thus relies on

several assumptions about the prior art landscape. First, KSR assumes a starting reference point or points in the art, prior to the time of invention, from which a skilled artisan might identify a problem and pursue potential solutions. Second, KSR presupposes that the record up to the time of invention would give some reasons, available within the knowledge of one of skill in the art, to make particular modifications to achieve the claimed compound. See Takeda, 492 F.3d at 1357 ("Thus, in cases involving new chemical compounds, it remains necessary to identify some reason that would have led a chemist to modify a known compound in a particular manner to establish prima facie obviousness of a new claimed compound."). Third, the Supreme Court's analysis in KSR presumes that the record before the time of invention would supply some reasons for narrowing the prior art universe to a "finite number of identified, predictable solutions," 127 S. Ct. at 1742. In Ortho-McNeil Pharmaceutical, Inc. v. Mylan Laboratories, Inc., 520 F.3d 1358, 1364 (Fed. Cir. 2008), this court further explained that this "easily traversed, small and finite number of alternatives . . . might support an inference of obviousness." To the extent an art is unpredictable, as the chemical arts often are, KSR's focus on these "identified, predictable solutions" may present a difficult hurdle because potential solutions are less likely to be genuinely predictable."

It is respectfully stated that in the present case, there was no insinuation let alone predictable result, that Fuhrman's extract might be helpful in treating the claimed risk factors. Moreover, as noted in paragraph 9 of the Aviram declaration, even Mr. Aviram who was a co-author of the Fuhrman article, indicates that he was surprised to find later on that Fuhrman's extract reduces systolic blood pressure and plasma lipid levels.

Thus, the Examiner's rejection fails the obvious tests set by KSR.

Since the claims refer to overlapping risk factors, appellants will argue the risk factors seperately and identify where each one of the factors is claimed. In the following, each risk-factor or condition is related to briefly, the arguments below are supported in the Vaya and Aviram affidavits as referred to below:

1. Blood pressure or hypertension (claims 1, 2, 9, 10 and 28).

Hypertension is known as a factor that increases the risk for atherosclerosis. Therefore, reducing blood pressure may be helpful in preventing or alleviating atherosclerosis. The teachings of Fuhrman et al. could be considered obvious in view of teachings of the present application, but not vice versa. The fact that the end-result (atherosclerosis) may be treated by the licorice extract does not teach or render obvious

the finding that the cause (hypertension) may also be treated with the same agent. Reference is also made to the Aviram declaration, specifically to paragraph 9 thereof.

Patients suffering from both atherosclerosis and hypertension are usually treated with two different agents, each to treat one of the conditions. Even if there are some medicaments that treat both conditions, it is not at all obvious that Fuhrman's licorice extract is one of them and no evidence has been provided by the Examiner that this would have been obvious.

Furthermore, patients that suffer from atherosclerosis not necessarily suffer from hypertension. To support this statement, Appellants previously cited an article named "Endothelium-mediated Coronary Blood Flow Modulation in Humans", published at J. Clin. Invest. 92 (August 1993) pages 652-662. A copy of the article is also attached herewith and marked Annex C. The article describes a group of 12 people "with evidence of early **atherosclerosis** but normal cholesterol levels and **normal blood pressure**" (see abstract) [emphasis added]. Therefore, treating people that suffer from atherosclerosis with the claimed licorice extract is inherently different from treating the same extract people that might also suffer from hypertension.

Finally, the Fuhrman's licorice extract is not known to, nor has it been shown by the Examiner to, include any constituent which is known in the art to be effective against hypertension. Therefore, a skilled person would not have any reason to expect the extract to have the claimed efficacy and would not have a reasonable expectation of success in treating hypertension. This is also supported in the Aviram affidavit, where he states in paragraphs 9 and 10 that he was surprised to find, at a later date, that ethanolic licorice extract is in fact effective to reduce systolic blood pressure.

2. Lowering total cholesterol levels and LDL levels (claims 23, 24, 25 and 31).

Reducing the susceptibility of LDL to oxidation, which is described by Fuhrman is expected to result in having less oxidized LDL. Since oxidized LDL is not found in the blood, but rather sticks to the blood-vessel walls, reducing LDL oxidation could result in leaving *more* LDL in the blood, and thus, increasing, rather than decreasing, its blood concentration. Thus, Fuhrman teaches away from trying to treat these conditions with her licorice extract.

Furthermore, Fuhrman herself teaches that administering the licorice extract to humans resulted in no significant change in cholesterol, this is also supported in the Aviram affidavit, paragraph 5. It was only the inventors of the present application, who

surprisingly found that, cholesterol ***is*** lowered in *hypercholestrolemic* patients, against what would be expected in view of Fuhrman. In fact, this was non-obvious enough, so that Fuhrman herself published, in 2002, another research, titled "Antiatherosclerotic Effects of Licorice Extract Supplementation on Hypercholesterolemic Patients: Increased Resistance of LDL to Atherogenic Modifications, Reduced Plasma Lipid Levels, and Decreased Systolic Blood Pressure". A copy of the article, published in Nutrition, 18 (2002) 268-273, was previously submitted by appellants and is attached herewith, marked Annex D.

There is no reason to believe that an agent effective in reducing the susceptibility of LDL to oxidation will also be effective in reducing LDL levels or total cholesterol levels, nor was such a reason provided by the Examiner. The above mentioned article teaches that the 12 atherosclerotic people had normal cholesterol levels, thus, treating cholesterol levels is inherently different from treating atherosclerosis.

3. **Lowering LDL levels and triglycride levels (claims 1, 23, 25, 26, 30 and 32) without decreasing HDL level (claim 8).**

Appellants fail to understand how the disclosure of Fuhrman renders any of these effects obvious, and fail to find an explanation in the Office Action. It is submitted that reducing LDL susceptibility to oxidation was not known to have any effect on these risk factors and is in fact irrelevant thereto. In fact, lowering LDL susceptibility to oxidation would be expected to raise the blood LDL levels, since oxidation removes some of the LDL.

4. Lowering glucose concentration in the blood (claims 1, 5), treating chronic renal failure (claims 9, 10 and 29).

Appellants fail to understand how the disclosure of Fuhrman renders any of these effects obvious, and fail to find an explanation in the Office Action. It is submitted that reducing LDL susceptibility to oxidation was not known to have any effect on these risk factors and is in fact irrelevant thereto.

In view of the above arguments it is submitted that a person of ordinary skill in the art, reading Fuhrman, would avoid using its extract to treat any of the risk factors recited in the claims of the present application.

B. Sha teaches against using an extract such as that used by Fuhrman for treating the claimed risk factors and conditions.

Sha uses a water soluble licorice extract for treating the risk factors. Sha specifically states that an active ingredient in licorice is glyzirrhiznic acid (or

glyzyrrhizine), See Col. 3, lines 41-47 in Sha. . Thus, if a person of ordinary skill in the art would have looked for a replacement for the licorice extract he would have searched for a different extract containing glyzirrhiznic acid. It is against the teachings of Sha to use a material not including glyzirrhiznic acid, let alone to replace his extract with such an extract.

Fuhrman states that the water insoluble licorice extract she uses is free of said acid. Accordingly, it is against common sense to use Fuhrman's extract, which is not free of glyzirrhiznic acid for the treatment of Sha's risk factors, which Sha specifically states are a part of his extract. In this regard, appellants also refer to Prof. Vaya's declaration, paragraphs 12 and 14.

Appellants have raised this argument in previous responses and the Examiner was not convinced thereby and states on page 5 of the final action: "The Examiner is not persuaded by the Argument that Sha et al teaches away from using Fuhrman's extract simply because different active ingredients have been identified because a plant extract is useful whether its active ingredients have been identified or not."

Appellants are confused by this argument. It is not a question of whether the active ingredients have been ***identified***, the active ingredients are simply ***not present***. Sha's active ingredient is identified as glyzirrhiznic acid. ***Glyzirrhiznic acid is not present in Fuhrman's extract.*** Furthermore, if it has not been identified (even if present), as appears to be admitted by the Examiner, this would destroy any motivation for using the extract of Fuhrman for this condition.

In addition, Fuhrman mentions glabridin as being an active ingredient. As explained in detail in Prof. Vaya's declaration, paragraphs 6, 7 and 13. Glabridin is not present in the water soluble extract of Sha.

The Examiner further states that because Sha teaches that in soluble form, glyzirrhiznic acid is an active ingredient, there would be a desire to provide a water insoluble form which is also active (page 4, paragraph 2 of the action). Appellants respectfully disagree. This statement assumes that there is something wrong with water-soluble forms, which would motivate a skilled person to look for insoluble forms. But the Examiner didn't show that there are any references, market forces, or design needs that would motivate replacing a water soluble ingredient with a water-insoluble one. It is noted, that the Vaya declaration filed by appellants, shows the contrary, that it would be against common sense to do so, see paragraphs 11, 17 and 19. Furthermore, even if it might be argued that it would be obvious to use a water insoluble form of glyzirrhiznic

acid, what reason would there be to use a water insoluble extract that does not include the acid?

Even if arguendo there was such motivation, there was nothing that could have pointed the skilled person towards Fuhrman's extract, or to any other particular extract. The possibilities of possible extracts that are at least as obvious as that of Fuhrman are unlimited. Finally, Fuhrman's extract is the less likely of the unlimited possibilities, because it is positively known to be free of the ingredient believed to be active in Sha, and because there are teachings against using it, as discussed above.

The Examiner's rejection can be interpreted as hindsight only. Since the water insoluble extract claimed was considered to have no effect on the claimed risk factors at the time of the invention, there would be neither desire nor logic in using the water insoluble claimed extract for treating Sha's risk factors. At most, there would be a desire to find another extract having glyzirrhiznic acid.

The Examiner also states that a person of ordinary skill would prefer the use of water insoluble extracts of Fuhrman since these extracts have a longer shelf life (page 4, paragraph 1 of the action). This reason is presented without any proof. Appellants respectfully disagree and submit, as evidence by Prof. Vaya in paragraph 17 of his attached declaration, that using a water insoluble extract has no effect on the shelf life thereof. One could expect such motivation to be effective only if the compounds were similar or were shown to have similar efficacy. By itself, the Examiner's suggested motivation would not motivate one to replace one compound which was found to be effective by another which was not found to be effective.

The only common feature that might be found between the two references is that they are both extracts of licorice. However, a person of ordinary skill is well aware that different extracts, specifically water soluble and water insoluble extracts, have widely different constituents and should be expected to have different effects. Considering there is no motivation to replace the extracts, and considering the reasons to abstain from doing so, appellants submit that the rejection is at odds with common sense, and with standard practice. As evidence thereof, appellants resubmit the declaration by Prof. Vaya.

While the extract disclosed in Fuhrman and that disclosed in Sha are both derived from licorice, it is evident from the references and as stated above, the extract of Sha and the extract of Fuhrman are completely different and there is no evidence or

teaching that the extract of Fuhrman would be useful in treating the conditions of Sha. To a person of skill in the art a water soluble licorice extract and a water insoluble licorice extract could be expected to be as different from each other as extracts from different materials, since most or all of the components would be different.

Claims 7, 11, 13-17, 19-22, 27 and 33 depend directly or indirectly from claims 1, 9, 10 and 23 and are each considered to distinguish over the cited reference, in any combination, for at least the same reasons given in support thereof.

At least some of the claims add further patentable weight to the independent claims. For example, claim 33 depends on claim 1 and recites "wherein administering to said patient an effective amount of a licorice extract consists of administering to said patient a pharmaceutical or nutraceutical preparation, said preparation being free from water-soluble licorice extract."

The Examiner has not provided a specific rejection to claim 33. Appellants submit that as stated above, Sha teaches against using Fuhrman's extract to treat his stated risk factors, let alone replacing his extract with Fuhrman's extract. Sha's extract is a water soluble extract with glyzyrrinzic acid as an active ingredient. Fuhrman's extract is a water insoluble extract free of glyzyrrinzic acid. Accordingly, it would be against the object of Sha and Fuhrman to administer a preparation being free from water soluble licorice extract to treat the risk factors recited in claim 1.

Conclusion

Claims 1, 2, 5, 7-11, 13-17 and 19-33 are believed to patentably distinguish over Fuhrman and Sha, in any combination, for at least all of the above reasons. Therefore, it is respectfully requested that the Board reverse the Examiner's final rejection for those claims.

Appellants are separately arguing the patentability of each of claims 1, 2, 5, 8-10, 23-26 and 28-33, since that re differentiated from each other either by the combinations of factors or conditions. Claim 33 further limits the preparation administere to be free of water soluble licorice extract. The other pending claims are patentable by virtue of their dependency.

Respectfully submitted,

by /[illegible]/ #41,016

Paul Fenster

Registration No. 33,877

Date: December 8, 2008

CLAIMS APPENDIX

The text of the claims involved in the appeal is as follows:

1. A method for lowering at least one risk factor in a patient suffering from said risk factor, comprising

identifying that said patient suffers from said at least one risk factor; and

administering to said patient an effective amount of a licorice extract which is water-insoluble and free from glycyrrhizinic acid, thereby lowering said risk factor, wherein said at least one risk factor comprises a risk factor selected from the group consisting of high blood pressure, high blood glucose concentration, and high blood triglycerides concentration.

2. The method of claim 1, wherein one of said at least one risk factor is high blood pressure.

5. The method according to claim 1, wherein one of said at least one risk factor is high blood glucose concentration.

7. A method according to claim 1, wherein said patient suffers from at least two risk factors, at least one of which is selected from the group consisting of high blood pressure, high blood glucose concentration, and high blood triglycerides concentration, and at least one selected from the group consisting of high LDL (low-density lipoprotein) susceptibility to retention, high LDL susceptibility to aggregation, high blood total cholesterol, high LDL levels, and high VLDL (very low-density lipoprotein) concentration.

8. A method according to claim 7, for treating a patient suffering from high blood triglycerides and high LDL (low-density lipoprotein) levels without decreasing the HDL (high density lipoprotein) level of said patient, comprising

administering to said patient an effective amount of a licorice extract which is water-insoluble and free from glycyrrhizinic acid, thereby treating the high blood triglycerides and high LDL levels without decreasing the HDL level of said patient.

9. A method for treating a patient suffering from at least one condition, comprising

identifying that said patient suffers from said at least one condition; and

administering to said patient an effective amount of a licorice extract which is water-insoluble and free from glycyrrhizinic acid, wherein said at least one condition is selected from the group consisting of hypertension and chronic renal failure.

10. A method for preventing a patient from suffering from at least one condition, comprising

identifying that said patient is in high risk to suffer from said at least one condition; and

administering to said patient an effective amount of a licorice extract which is water-insoluble and free from glycyrrhizinic acid, wherein said at least one condition is selected from the group consisting of hypertension and chronic renal failure.

11. The method according to claim 1, comprising obtaining said licorice extract in a method comprising extracting licorice in ethanol.

13. The method according to claim 7, comprising obtaining said licorice extract in a method comprising extracting licorice in ethanol.

14. The method according to claim 8, comprising obtaining said licorice extract in a method comprising extracting licorice in ethanol.

15. The method according to claim 9, comprising obtaining said licorice extract in a method comprising extracting licorice in ethanol.

16. The method according to claim 10, comprising obtaining said licorice extract in a method comprising extracting licorice in ethanol.

17. The method according to claim 1, wherein said licorice extract dissolves in ethanol.

19. The method according to claim 7, wherein said licorice extract dissolves in ethanol.

20. The method according to claim 8, wherein said licorice extract dissolves in ethanol.

21. The method according to claim 9, wherein said licorice extract dissolves in ethanol.

22. The method according to claim 10, wherein said licorice extract dissolves in ethanol.

23. A method for lowering at least one risk factor in a patient suffering from said at least one risk factor, comprising

identifying that said patient suffers from said at least one risk factor; and

administering to said patient an effective amount of a licorice extract which is water-insoluble and free from glycyrrhizinic acid, thereby lowering said risk factor, wherein said at least one risk factor comprises a risk factor selected from the group consisting of high blood total cholesterol, high blood LDL levels, high blood triglycerides concentration and high blood VLDL (very low-density lipoprotein) concentration.

24. The method according to claim 23, wherein one of said at least one risk factor is high blood total cholesterol.

25. The method according to claim 23, wherein one of said at least one risk factor is high LDL levels.

26. The method according to claim 1, wherein one of said at least one risk factor is high blood triglycerides concentration.

27. The method according to claim 23, wherein one of said at least one risk factor is high blood VLDL concentration.

28. The method according to claim 9, wherein one of said at least one condition is hypertension.

29. The method according to claim 9, wherein one of said at least one condition is chronic renal failure.

30. A method for treating a patient suffering from at least one condition, comprising

identifying that said patient suffers from said at least one condition; and

administering to said patient an effective amount of a licorice extract which is water-insoluble and free from glycyrrhizinic acid, wherein said at least one condition comprises a condition selected from the group consisting of hypercholestoremia and hypertriglyceridemia.

31. The method according to claim 30, wherein one of said at least one condition is hypercholesterolemia.

32. The method according to claim 30, wherein one of said at least one condition is hypertriglyceridemia.

33. The method of claim 1, wherein administering to said patient an effective amount of a licorice extract consists of administering to said patient a pharmaceutical or nutraceutical preparation, said preparation being free from water-soluble licorice extract.

EVIDENCE APPENDIX

This appeal brief presents no additional evidence

RELATED PROCEEDINGS APPENDIX

This appeal has no related proceedings

356/02918

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Luba COHEN.
Serial Number:	09/955,933
Filed:	September 20, 2001
For:	LICORICE EXTRACT FOR USE AS A MEDICAMENT
Art Unit:	1651
Examiner:	Deborah K. Ware

DECLARATION OF MICHAEL AVIRAM UNDER USC 37 §1.132

Mail Stop AF
Honorable Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, the undersigned, Michael AVIRAM, a citizen of Israel, whose address is, The Lipid Research Laboratory, Rambam Medical Center, Bat Galim, Haifa, Israel 31096 , do solemnly declare, as follows:

1. I was named as co-author in an article titled "Licorice extract and its major polyphenol glabridin protect low-density lipoprotein against lipid peroxidatin: in vitro and ex vivo studies in humans and in atherosclerotic apolipprotein E-deficient mice" published in American Journal of Clinical Nutrition volume 66 (1997) pages 267-275 by the American Society for Clinical Nutrition (hereinafter Fuhrman et al).
2. My curriculum vitae is attached herewith as part of this declaration.
3. The scientific work described in Fuhrman et al was done under my supervision.
4. Fuhrman et al describes that an ethanolic extract of licorice reduced LDL susceptibility to oxidation, and reduced atherosclerotic lesion in atherosclerotic mice.
5. Fuhrman et al describes that the same ethanolic extract of licorice had no significant effect on blood cholesterol levels in healthy humans.
6. The component identified in Fuhrman et al to be active as an anti-oxidant was glabridin.
7. The chemical structure of glabridin does not resemble that of anti-hypercholesterolemic drugs known to me, neither does it resemble the chemical structure of anti-hypertension drugs known to me.

8. Fuhrman et al does not describe, and I am not aware of any scientific article that did describe, prior to the date of the application, any other constituent of ethanolic licorice extract, the chemical structure of which does resemble that of any of the above-mentioned drugs.
9. I was surprised to find, in performing another research, in hypercholesterolemic patients, conducted under my supervision, that ethanolic licorice extract is in fact effective to reduce systolic blood pressure. I was also surprised to find, in the same research, that ethanolic licorice extract reduces plasma lipid levels.
10. The findings mentioned under paragraph 9 above were published on 2002 in Nutrition, 18:268-273, under the title "atniatherosclerotic effects of licorice extract supplementation on hypercholesterolemic patients: increased resistance of LDL to atherogenic modification, reduced plasma lipid levels, and decreased systolic blood pressure".

The undersigned further declares that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true

December 24 2006
Date

M. Aviram
Michael AVIRAM

TECHNION – FACULTY OF MEDICINE

Date: November 2006

RESUME

1. PERSONAL DETAILS

MICHAEL AVIRAM

Professor, Head of the Lipid Research Laboratory, Technion Faculty of Medicine, Rappaport Institute for Research in the Medical Sciences and Rambam Medical Center, Haifa, Israel
http://hebrew.rambam.org.il/aviram
http://www.technion.ac.il/~medicine/faculty/lipid.htm
http://www.technion.ac.il/~rapinst/aviram.html
http://md.technion.ac.il/inner/department.php?department_id=50

Date & Place of Birth: December 7, 1948, Israel. (ID # 00557978-4)

Marital status: Married to Bruria, 3 children (Amitai, Yotvat and Rohtem).

Work Address: Lipid Research Laboratory, Technion Faculty of Medicine, and Rambam Medical Center, Haifa, Israel 31096

Tel: 972-4-8542970 Fax: 972-4-8542130 E mail: aviram@tx.technion.ac.il

Home address: 59 Haplugot St., Kiriat-Haim, 26253, Israel. (Tel: 972-4-8704587).

2. ACADEMIC DEGREES

1975-1978 **Doctorat Studies**, Clinical Biochemistry, Faculty of Medicine, Technion - Israel Institute of Technology, Haifa, Israel.
D.Sc. (Supervisor: Nobel Prize Laureate Prof. Avram Hershko).

1973-1975 **Graduate Studies**, Clinical Biochemistry, Faculty of Medicine, Technion -Israel, Institute of Technology, Haifa, Israel.
M.Sc. (Supervisor: Nobel Prize Laureate Prof. Avram Hershko).

1966-1970 **Undergraduate Studies**, Chemistry, Technion-Israel Institute of Technology, Haifa, Israel, **B.Sc.**

* Israel Defence Forces (1970-1973, final rank in Reserve – Leutenent Colonel)

1978- 1980 **Postdoctoral Fellow:** Arteriosclerosis Center, Massachusetts Institute of Technology **(M.I.T)**, Cambridge, MA, U.S.A.

3. ACADEMIC APPOINTMENTS

2004 – present **Professor of Biochemistry**, Technion Faculty of Medicine, Haifa, Israel

1991- 2004 **Associate Professor of Biochemistry**, Technion Faculty of Medicine, Haifa, Israel.

1997-1998 **Professor (Visiting), University of Michigan, Ann Arbor, MI, U.S.A.** "Paraoxonase, lipid peroxidation, and macrophage cholesterol accumulation: molecular and cellular biology" (with Prof. Bert La Du).

1991-present **Member**, The Rappaport Institute for Research in the Medical Sciences, Haifa, Israel.

1994-2000 **Deputy Director**, The Rappaport Institute for Research in the Medical Sciences, Haifa, Israel.

1987-1988 **Associate Professor (Visiting), Division of Metabolism, Department of Medicine, University of Washington, Seattle, WA, U.S.A.** "Lipase-modified LDL and macrophage cholesterol metabolism" (with Prof. Edwin Bierman and Prof. Alan Chait).

1986-1987 **Visiting Scientist, Specialized Center of Research (SCOR) in Atherosclerosis, Department of Medicine, Columbia University, New York, NY, U.S.A.** "Lipoprotein lipids modifications and macrophage cholesterol accumulation". (with Prof. Richard Deckelbaum).

1989-1997 **Adjunct Associate Professor**, Department of Food Engineering and Biotechnology, Technion, Haifa, Israel.

1985-1989 **Adjunct Assistant Professor**, Department of Food Engineering and Biotechnology, Technion, Haifa, Israel.

1984 **Visiting Scientist, Institute of Clinical Medicine, University of Tromso, Tromso, Norway** (3 months) "Dietary fatty acids, lipoproteins, and endothelial cell function". (with Prof. Arne Nordoy).

1983 **Visiting Scientist, Institute of Pharmacology, University of Milano, Milano, Italy.** "Plasma lipoproteins and platelet prostaglandins". (with Prof. Cesare Sirtori).

1973-1978 **Instructor**, Clinical Biochemistry, Faculty of Medicine Technion - Israel Institute of Technology, Haifa, Israel.

1975-1978 **Doctoral Studies,** "Turnover of the enzyme tyrosine-aminotransferase". Clinical Biochemistry, Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel. (Supervisor: Nobel Prize Laureate Prof. Avram Hershko).

1973-1975 **Graduate Studies,** "Degradation of RNA in cultured hepatoma cells". Clinical Biochemistry, Faculty of Medicine, Technion - Israel Institute of Technology, Haifa, Israel. (Supervisor: Nobel Prize Laureate Prof. Avram Hershko).

1966-1970 **Undergraduate Studies**, Faculty of Chemistry, Technion - Israel Institute of Technology, Haifa, Israel.

4. PROFESSIONAL EXPERIENCE AND DUTIES

Technion and Rambam Medical Center Activities

1980-present Head, Lipid Research Laboratory, Technion Faculty of Medicine, Rappaport Institute for Research in the Medical Sciences, and Rambam Medical Center.
1999-present Chairman, Department of Laboratory Medicine, Rambam Medical Center.
2006 Member of the Technion's Senate Harvey Prize Committee.
2005-present Member of the Technion's Senate Standing Committee and the Elected Senate.
2005-present Member of the Technion Academic Council for Continous and External Studies.
2004–present Member of the Technion's Senate Committee for Research.
1994-2005 Chief Scientific Advisor, Rambam Medical Center.
1993-2001 Member, Committee for Human Resources, Rambam Medical Center.
1994-2000 Deputy Director, Rappaport Institute for Research in the Medical Sciences.
1994-1997 Head, Committee for Teaching and Curriculum, Technion Faculty of Medicine.
1993-1996 Chairman, The Technion Committee for Promotion of Laboratory Technicians.
1994-1996 Member, Committee for Graduate Studies, Technion Faculty of Medicine.
1993-1994 Deputy Head, Committee for Research, Technion Faculty of Medicine.
1993-1994 Deputy Head, Committee for Teaching, Technion Faculty of Medicine.
1991-1994 Member, the Secretariat, Board of Medical Research, Israel Ministry of Health.

International and National Activities

2004-2007 Member of the Scientific Advisory Board and of the Executive Board of Governors , D- CURE, a non-profit Diabetes Research Organization.
2006 Chairman, Israel Science Foundation (ISF) Centers of Excellence Committee.
2004-present Member, the Editorial Advisory Board, *Drug Design Reviews – Online.*
2004-present Member, the Editorial Board, *Medicinal Chemistry Reviews.*
2004-present Member, the Editorial Board, *Israel Medical Association Journal.*
2004-2005 Guest Editor for the Paraoxonases Serial Reviews: Free Radical Biology & Medicine.

2002-2005	Member, Committee of Process for the assessment of Scientific support for Claims on Food (PASSCLAIM, Group on Diet related atherosclerosis).
2001-2002	Member, the Ministry of Health Committee for Continuous Education Reward (Gemul Hishtalmut).
2001-2005	Member, the Scientific Advisory Board, Lycopin Fourm. Germany
2000-2005	Scientific Advisor, Roche Diagnostics GmbH, Penzberg, Germany.
2000-2005	Member, the Scientific Advisory Board (SAB), Molecutec and PharmaVitae, Molecular Technology Corporation, New York, NY, USA.
2000-2005	Member, the Scientific Advisory Board (SAB), Esperion Therapeutics, Ann Arbor, MI, USA.
2000-2005	Member, the Scientific Advisory Board (SAB), BioPreventive, Noninvasive Biomedical Diagnostic Tools, Migdal-Haemek, Israel.
2000-2005	Member, the Scientific Advisory Board (SAB), Profile Advanced Technologies (PAT), Jerusalem, Israel.
2000-present	Member, the Editorial Board, *Current Medicinal Chemistry.*
1998-present	Member, the Scientific Advisory Board (SAB) ROLL International, PomWonderful, LA, CA, USA.
1996-present	Member, the Editorial Board, *Czynniki Ryzyka.*
1985-1989	Editor-in-Chief, Israel Journal of Clinical Biochemistry and Laboratory Sciences.
1981-1993	Member, the Secretariat, Israel Society for Clinical Biochemistry (ISCB).
1981-1986	Member, the Secretariat, Israel Biochemical and Microbiological Union (IBMU).
1980-1986	Member, the Secretariat, Israel Biochemical Society (IBS).
1970-1973	Israel Defence Forces. Final rank in reserves – Colonel.

*** REVIEWER FOR ALL MAJOR INTERNATIONAL JOURNALS ON ATHEROSCLEROSIS.**

5. RESEARCH INTERESTS

General Goal: Study of Mechanisms involved in Macrophage Cholesterol accumulation and Foam Cell formation under Oxidative Stress during Atherogenesis: Role of Dietary Antioxidants and of Paraoxonases.

Specific Areas of Research:

1. Lipoproteins Oxidation and Atherosclerosis (1980 – present)

Macrophage – mediated oxidation of LDL and foam cell formation are the hallmark of early atherogenesis. We were the first to demonstrate the role of cellular oxygenases (such as NADPH oxidase) and of antioxidants (such as the glutathione system) in cell - mediated LDL oxidation and in atherosclerotic lesion development.

2. Dietary Antioxidants and Atherosclerosis (1990 – present)

We have provided evidence, for the first time, that the inhibitory effect of some flavonoid antioxidants on macrophage – mediated LDL oxidation (and on atherosclerosis development) is related to the polyphenols interaction with the lipoprotein directly, as well as to their accumulation in arterial macrophages.

3. Paraoxonases, Lipid Peroxidation and Atherosclerosis (1997 – present)
Recently, we provided evidence that HDL- associated Paraoxonase can hydrolyze oxidized lipids in oxidized lipoproteins,macrophages, and in atherosclerotic lesions. Paraoxonase thus may act as a second line of defense against oxidative stress and atherosclerosis development.

6. TEACHING EXPERIENCE

1992-present
Chairing and teaching **basic courses** to Medical Students.
1. Chair and lecturer: **Biochemistry** to Medical Students (Course # 274226).
2. Chair: **Biochemistry Laboratory** (Course # 274227).
3. Lecturer: **Clinical Biochemistry** to Medical Students (Course # 276310).

1981-present
Chairing and teaching in **elective courses** to Medical and Graduate Students:
1. **Lipoproteins and Atherosclerosis** (Course # 277426).
2. **Lipids and Lipoprotein Metabolism** (Course # 068318).

* Teaching: **Clinical Biochemistry** to undergraduate students, Faculty of Life Sciences, Bar Ilan University, Ramat-Gan, Israel (1992-1999).

2002-present - Technion Permanent Outstanding Lecturer
Outstanding teacher, Faculty of Medicine: every year since 1993

2001 - Faculty of Medicine, Outstanding Lecturer. Score – 4.76 (out of 5.00).
2000 - Muriel and David Jacknow Award for Excellence in Teaching.
2000 - Faculty of Medicine, Outstanding Lecturer. Score – 4.71.
1999 - Faculty of Medicine, Outstanding Lecturer. Score - 4.76.
1997 - Faculty of Medicine, Outstanding Lecturer. Score - 4.64.
1996 - Faculty of Medicine, Outstanding Lecturer. Score - 4.70.
1995 - Faculty of Medicine, Outstanding Lecturer. Score - 4.67.
1994 - Faculty of Medicine, Outstanding Lecturer. Score - 4.64.
1993 - Faculty of Medicine, Outstanding Lecturer. Score - 4.70.
1993 - Technion President Award : Best Lecturer of the Year.

7. MEMBERSHIP IN PROFESSIONAL SOCIETIES

National

Israel Society for Atherosclerosis.
Israel Society for Diabetes.
Israel Society for Laboratory Medicine (previously - Clinical Biochemistry).
Israel Biochemical Society.
Israel Society for Oxygen and Free Radical Research.

International

American Society for Biochemistry and Molecular Biology (ASBMB).
Academy of Clinical Laboratory, Physicians and Scientists.
American Heart Association. Council on Arteriosclerosis.
American Association for Clinical Chemistry.
American Federation for Clinical Research.
Biochemical Society.
European Society for Clinical Investigation.
European Atherosclerosis Society.
European Lipoprotein Club.
International Atherosclerosis Society.
International Society for the study of Fatty acids and Lipids (ISSFAL).
International Society for Free Radical Research (ISFRR).
International Society on Thrombosis and Haemostasis.

8. HONORS

2002 - Technion Permanent Outstanding Lecturer Award
Outstanding teacher, Faculty of Medicine every year since 1993
1998 - Pfizer Lecturer Award, Clinical Research Institute of Montreal, Canada ("LDL oxidation and atherosclerosis").
1998 - NIH, Office of Alternative Medicine (OAM),Symposium Award ("Antioxidants, LDL oxidation and atherosclerosis").
1994 - Senior Investigator International Prize for Research on Oxidation of Lipoproteins: The ARCOL Prize, Pastuer Institute, Lille, France ("Macrophage-mediated oxidation of LDL").
1994 - Faculty of Medicine Award for Scientific achievements.
1993 - Faculty of Medicine Award for scientific achievements.
1993 - Servier Investigator Award for Research on Antioxidants. European Society on Free Radicals Research, Valencia, Spain.
1987 - Fogarty International Fellowship (Seattle, WA, USA). "Lipids-modified LDL".

9. STUDENTS INSTRUCTION

D.Sc. / Ph. D. Theses

1. Bianca Fuhrman: "Platelet secretory products and macrophage lipoprotein metabolism". 1988-1992.
2. Melia Paizi: "The possible role of fibronectin in plasma cell dyscrasia related problems."1986-1990 (Co- supervisor with Prof. G. Spira).

3. Qianmei Li: "Macrophage metabolism of the fatty acids in the LDL cholesteryl ester". 1993-1995.
4. Paula Belinky: "Licorice as an inhibitor of LDL oxidation". 1994-1998.
5. Irit Maor: "Relationships between oxidation and aggregation processes in LDL

derived from apolipoprotein E-deficient mice during atherogenesis". 1995-1999.

6. Marielle Kaplan: "Proteoglycans and macrophage uptake of oxidized LDL" 1996-2000.
7. Andrea Szuchman: "Chemical markers for oxidative stress in biological systems". 2001-2005.
8. Orit Rosenberg (Grunfeld): "Paraoxonase 1 overexpression and protection against atherosclerosis" 2003-2006.
9. Maayan Sheiner (Ben-dor): "Paraoxonase 2 regulationin in macrophages:signal transduction and transcription factor pathways" 2004-2007.
10. Hagai Tavori : "Paraoxonase protection against oxidative stress". 2007-2010.

M.Sc. Theses

1. Yaakov Berkovitz: "Platelet adhesion in whole blood". 1979-1981 (Co-supervisors: Profs Marmur and Brook).
2. Idit Bornstein: "Platelet adhesion". 1981-1983 (Co-supervisors: Profs Marmur and Brook).
3. Bianca Fuhrman: "The effect of chylomicrons on platelet function". 1983-1985 (Co-supervisor: Prof. Brook).
4. Nechama Segal: "Surfactants and platelet aggregation". 1984-1986 (Co-supervisors: Profs Marmur and Brook).
5. Edna Hochgraff: "The effect of lovastatin on platelet function, composition and fluidity in hypercholesterolemic patients". 1988-1990 (Co-supervisor: Prof. Cogan).
6. Irit Maor: "Platelet secretory products and macrophage cholesterol metabolism". 1990-1992.
7. Judith Oiknine: "The effect of macrophage activation on the uptake of LDL". 1991-1993.
8. Mira Rosenblat: "Macrophage oxidation of LDL". 1993-1995.
9. Limor Ben Yaish: "The effect of lycopene on macrophage cholesterol metabolism". 1995-1997.
10. Orit Grunfeld: "Paraoxonase and macrophage foam cell formation". 2000-2002.
11. Anat Katzir: "Oxidized LDL and macrophage MAP kinase signal transduction". 2000-2002 (Co-supervisor: Prof. Polack).
12. Maayan Ben-Dor: "Macrophage maturation and foam cell formation". 2002-2004.
13. Michal Efrat : "HDL phospholipids and paraoxonase 1 activities". 2006 – 2008.

Undergraduate Students

1. Nir Lubetchky - "Macrophage lipid peroxidation". 1992-1993.
2. Limor Ben Yaish - "Cell membrane lipid peroxidation in macrophages". 1994-1995.
3. Shlomi Buch - "Antioxidative properties of Licorice against LDL oxidation". 1994-1996.
4. Beha Francis - "Macrophage foam cell formation under oxidative stress". 1999-2000.
5. Ayelet Partush – " Acetyl Choline Esterase hydrolyzes lipid peroxides". 2004-2005.

6. Roni Oren – " LPC attenuates macrophage - mediated oxidation of LDL". 2005-2006.
7. Yasmin Chativ – "Macrophage paraoxonase 2 (PON2) regulation by the urokinase plasminogen activator (uPA) system". 2005-2006.
8. Orly Sapir – "Glucose destabilizes HDL – associated paraoxonase1 (PON1); implications to Diabetes". 2005-2006.

M.D. Theses

1. Ron Diukman: "The effect of soy protein diet on plasma lipoproteins in rabbits". 1977-1978.
2. David Ron: "HDL in the elderly". 1978-1979.
3. Ema Shilansky:"HDL in atherosclerotic patients". 1978-1979.
4. Michael Lanchet: "HDL in young males after myocardial infarction". 1979-1980.
5. Yitzchak Sarugo: "The effect of HDL on platelet function". 1980-1982.
6. Arthur Veschler: "The effect of carnitineon platelet function and plasma lipoproteins in patients with chronic renal failure ohemodialysis". 1980-1982.
7. Asher Shmulevitz: "Platelets LDL receptor". 1982-1983.
8. Eyal Herzog: "saturated fat rich diet on platelet function". 1982-1984.
9. Ron Hoffman: "Plasma lipoproteins in celiac disease". 1987-1988.
10. Eias Kasem: "Dietary olive oil and LDL oxidation in humans". 1991-1992.
11. Danniel Karter: "Macrophage-lipid peroxidation by PMA". 1994-1996.
12. Lena Koren: "Monocyte-macrophage differentiation under oxidative stress". 1999-2000.
13. Nir Shimoni : "High serum HDL and cardiovascular diseases". 2001-2002.
14. Ayelet Partush : "Oxidized LDL and macrophage foam cell formation". 2005-2006.
15. Rony Oren : "Lysophosphatidylcholine and macrophage –mediated oxidation of LDL". 2005-2006 .

Residents (Basic Science)

1. Gideon Derayfus- Carmel Hospital, Haifa: "The effect of dietary vegetarian proteins on plasma lipoproteins in rabbits". 1979-1980.
2. Ruth Baruch (Gershoni)- Rambam Hospital, Haifa: Plasma lipoprotein pattern in young children 1980-1981.
3. Jacob Baruch-Rambam Hospital, Haifa: Dyslipoproteinemia in primary biliary chirrosis". 1980-1981.
4. Avi Viener-Rambam Hospital, Haifa: "The effect of plasma lipoproteins on platelet function in hypercholesterolemic patients". 1981-1982.
5. Giora Winterstein- Rambam Hospital, Haifa: "Prostaglandin pathway and platelet function in hypercholesterolemia". 1982-1983.
6. Nassara Chalil- Ziv Hospital, Zefat: "Plasma fatty acid after fat rich meal". 1983-1984.
7. Efrat Wolfovitz - Rambam Hospital, Haifa, "Macrophage lipid metabolism". 1984-1985.
8. Mondir Boulos - Rambam Hospital, Haifa: "The effect of plasma lipoproteins derived from hypercholesterolemic patients on macrophage cholesterol content". 1985-1986.

9. Lavi Klein-Rambam Hospital, Haifa: "Plasma lipoprotein fluidity in hypercholesterolemic patients". 1987-1988.
10. Ron Hoffman - Rambam Hospital, Haifa: "Anti oxidation properties of hypocholesterolemic drugs". 1990-1991.
11. Rafi Azugi - Ziv Hospital, Zefat: Effect of estrogen on plasma lipoprotein pattern. 1990-1991.
12. Osamah Hussein - Ziv Hospital, Zefat: "The effect of platelet secretory products on macrophage cholesterol metabolism in mouse peritoneum". 1990-1991.
13. Peter Barta - Rambam Hospital, Haifa: "Myocardial infarction and plasma lipid peroxidation". 1993-1994.
14. Yanir Kashif - Naharia Hospital, Naharia: "Xanthelasma and oxidized lipids". 1993-1994.
15. Sorina Schlesinger - Ziv Hospital, Zefat: "Fluvastatin and LDL oxidation". 1995-1996.
16. Gavriella Friedman-Ziv Hospital, Zefat: "Cholestyramine and LDL oxidation: 1995-1996.
17. Imad Sachnin: "Angiotenisn II and macrophage cholesterol metabolism". 1998-1999.
18. Ayelet Raz – "Regresion of atherosclerosis in apo E deficient mice by treatment with ACE inhibitors" – 1999-2000.
19. Shadi Hammood: "Effect of ACE inhibitors on oxidative stress are atherosclerosis in E^0 mice". 2001-2002.
20. Chitam Hussein – "Paraoxonase 2 in macrophages from hypercholesterolemic patients; effect of statin therapy". 2003-2004.
21. Alex Strizevski – "Human monocyte-macrophage ACE2 expression in hypertensive patients" 2004-2005.
22. Ronnen Saltz – "Macrophage atherogenicity in diabetes" 2004-2005.
23. Orna Nitzan – "Monocyte-macrophage differentiation under oxidative stress". 2005-2006.
24. Ido Bogner -"HDL composition affects its biological function and atherosclerosis ". 2006-2007.

Collaborating Senior Physicians

1. Shlomo Keidar, M.D.: "Angiotensin II, LDL oxidation and atherosclerosis". Rambam Medical Center, Haifa, 1988 - present.
2. Tony Hayek, M.D.: The atherosclerotic apolipoprotein E-knockout mice as a model to study lipoprotein modifications in atherosclerosis". Rambam Medical Center, Haifa, 1993 - present.
3. Osamah Hussein, M.D.: "Antioxidants against LDL oxidation and atherosclerosis". Ziv Hospital, Zefat, 1993 - present.
4. Raanan Shamir : "Intestinal paraoxonases". Rambam Medical Center, Haifa, 2001-2004.
5. Alexandra Lavy, M.D.: "Lipoprotein oxidation in atherosclerotic patients". Bnei Zion Hospital, Haifa, 1988 - 2000.
6. Yishai Levy, M.D.: "Carotenoids as antiatherogenic agents", Rambam Medical Center, Haifa, 1988 - 2000.
7. Avishay Elis, M.D.:"The effect of lycopene on macrophage cholesterol metabolism and atherosclerosis". Meir Medical Center, Kefar-Saba, 1996 - 2001.
8. Hanna Mandel, M.D.: "Peroxisomes and cholesterol metabolism" Rambam Medical Center, Haifa 1991-2000.

9. Reuven Bergman, M.D.: "Xanthelasma, Xanthogranulomatosis and cholesterol metabolism". Rambam Medical Center, Haifa, 1993 - 2000.
10. Daiana Gaitini, M.D. : Intima-Media Thickness (IMT) in atherosclerotic patients: effect of dietary antioxidants". Rambam Medical Center, Haifa, 2000-2004.

10. RESEARCH GRANTS

Academic

2005-2008 **Rappaport Institute Research Grant** - " Paraoxonases (PONs), Oxidized lipids and foam cell formation". - $60,000

2005-2007 **Israel Ministry of Science and Technology** – "Metabolic networks in pomegranate fruit: an analytical platform for food functionality" - $200,000 (together with Holland D., Neve Yaar Research Center and Amir R., MIGAL Research Center).

2004-2007 **The Israel Science Foundation (ISF, The Israel Academy of Sciences and Humanities)** – "The search for endogenous substrates of paraoxonase" - $90,000 (together with Dr J. Vaya., MIGAL Research Center).

2004-2006 **D-Cure Diabetes Research Grant** -"Paraoxonase (PON) substrates metabolic pathways under oxidative stress: studies under the diatetic environment" - $130,000 (together with Dr J. Vaya., MIGAL Research Center).

2004-2006 **The Niedersachsischen Minesteriums fur Wissenchaft and Kultur** - "Regulation of urokinase plasminogen activator (uPA) expression in monocytes during their differentiation into macrophages: consequences on atherogenesis in relation to macrophage-foam cell formation and to vascular smooth muscle cells migration and proliferation". – EU 100,000.

2005-2006 **Israel Ministry of Health** – "Paraoxonase in hypercholesterolemic patients" – $20,000.

2004-2007 **The Israel Science Foundation (ISF, The Israel Academy of Sciences and Humanities)** – "Regulation of macrophage atherogenicity by the haptoglobin polymorphism". - $200,000 (Cooperating Investigator. PI - Dr. A. Levy).

2002-2004 **Rappaport Institute Research Grant** - " Paraoxonases, Oxidized lipids and foam cell formation". - $75,000.

2001-2004 **Michigan Life Science Corridor Grant** -"Can Paraoxonase be used to treat Endotoxemia and Sepsis?" - $1,740,000 (PI – Dr. Bert La Du Aviram's part - $30,000, acting as an advisor to the University of Michigan, Department of Pharmacology Research Team.).

2000-2002 **The Niedersachsischen Minesteriums fur Wissenchaft and Kultur** – "Role of angiotensin II in macrophage cholesterol accumulation, foam cell formation and the induction of cytokines, in the progression of atherogenesis". – DM 200,000.

1998-2000 **Rappaport Institute Research Grant** - "Oxidized LDL and foam cell formation". - $120,000.

1997-2000 **The Israel Science Foundation (ISF , The Israel Academy of Sciences and Humanities)** - "Coronary heart disease: genetic, environmental and behavioral determinants. - $ 100,000 (PI - Dr. Jeremy Kark).

1995-1998 **The Israel Science Foundation (The Israel Academy of Sciences and Humanities)**- "Proteoglycans and macrophage uptake of oxidized LDL- $ 150,000.

1995-1997 **Rappaport Institute Research Grant** - "Oxidized LDL and foam cell formation". -$120,000.
1993-1995 **Israel Ministry of Health** "Macrophage lipids peroxidation " - $ 35,000.
1991-1994 **Rappaport Institute Research Grant-** "Oxidized LDL and foam cell formation". -$ 120,000.
1991-1994 **German (BMFT) - Israel Binational Grant**. "Platelet secretory products and macrophage cholesterol metabolism". - DM 250,000 (Co-PI with Dr. J.G. Brook).
1989-1991 **Israel Ministry of Health** - "LDL-platelet interaction" - $ 25,000.
1987-1988 **Fogarty International Fellowship** (Seattle, WA, USA). "Lipids - modified LDL". - $ 40,000.
1984-1987 **GSF, German (BMFT) - Israel Binational Grant**. "Platelet-modified LDL". - DM 200,000 (Co-PI with Dr. J.G. Brook).
1983-1985 **Israel Ministry of Health** "Lipoprotein-platelet interactions" - $ 25,000.
1984 **Norway - Israel Fellowship**. "Prostaglandin - lipoprotein interactions".
1983 **Italian - Israel Fellowship**. "Platelet prostaglandins and lipoproteins".
1981-1983 **Israel Ministry of Health** "Triglyceride and platelet function" - $20,000.
1978-1980 **Israel Ministry of Health** "Lipoprotein abnormality in atherosclerosis" - $20,000.

Commercial

2006-2008 **Ortho-Clinical Diagnostics (OCDUS) / Johnson & Johnson** – " Development of new serum paraoxonase (PON1) tests for atherosclerosis" - $356,000 (CO-PI with Dr D. Tawfik).
2006-2007 **Your Energy Systems** – "Liposomal Glutathione and atherosclerosis"- $70,000.
2006-2007 **Goya Holding S.A. (Genius s.r.l.)** – " Polyphenols enriched olive oil"-$ 15,000.
1998-2008 **Roll International Ltd.** – "Nutritional antioxidants, LDL oxidation and atherosclerosis" - $ 350,000.
2001-2005 **Roche Diagnostics GmbH** – "Serum paraoxonase and atherosclerosis" – DM 300,000.
2001-2003 **Pharmacia**– "Aldosterone,oxidative stress and atherosclerosis" - $ 75,000.
1995-2001 **Lycored (Makhteshim)**-"Lycopene and LDL oxidation."$ 180,000.
1998-1999 **Transphyto Ltd.** - "Dietary antioxidants and atherosclerosis" – FF 120,000.
1997-1998 **Dalidar Pharma Ltd.** "Ginger-derived polyphenols and atherosclerosis"- $ 50,000.
1995-1997 **Sandoz Pharma AG.** -"HMGCoA reductase inhibitors". - $ 50,000.
1994-1997 **Fertilizers and Chemicals** -"Licorice as an antioxidant". - $100,000.
1991-1993 **Bristol-Myers Squibb Grant**. "Hypocholesterolemic therapy and lipoprotein oxidation". - $50,000 (Co-PI with Dr. J.G. Brook).
1990-1992 **Merck, Sharp and Dohme Grant**. "Lipid-modified LDL and hypocholesterolemic drugs". - $ 80,000 (Co-PI with Dr. J.G. Brook).

Technion Research grants

1994-present Mechanisms of lipoproteins atherogenicity- $2500-$5000/year.

11. CONFERENCES - International (Invited Speaker)

2006-

Aviram M. "Wine flavonoids, LDL cholesterol oxidation and atherosclerosis" The Universe of Winw and Health, March 9-12, **Firenze, Italy.**

Aviram M. "Paraoxonase 1 (PON1) attenuates, macrophage foam cells formation". Paraoxonases and oxidative stress. University of Mainz, March 13, **Mainz, Germany.**

Aviram M. "Dietary antioxidants protects against cardiovascular diseases: the pomegranate example" International Congeress on Thrombosis, May 14-16, **Tel Aviv, Israel.**

Aviram M. "Atherosclerosis, diabetes and it complications" June 7-10, **Iasi, Romania.**

Aviram M. "HDL-associated paraoxonase 1 (PON1) attenuates lipoprotein oxidation, macrophage foam cells formation and atherosclerosis development" International Symposium on Atherosclerosis, June 18-22, **Rome, Italy.**

Aviram M. "Paraoxonase and macrophage foam cell formation" Second International Conference on Paraoxonases, September 7-10, **Debrecen, Hungary.**

Aviram M. "Pomegranate and Cardiovascular health". 6th International Phytochemical Conference Themes and Topics Phytochemicals: Aging and Health". October 16-17, **Buena Park, CA, USA.**

Aviram M. "Antioxidant properties of olive oil against LDL oxidation and atherosclerosis development". 28th ESPEN Congress Clinical Nutrition and Metabolism. Octorber 19-22, **Istanbul, Turkey.**

Aviram M. "Naturitional antioxidants protect against atherosclerosis: role for HDL-associated paraoxonase" Fourth international conference on mechanisms of action of nutraceuticals (ICMAN 4), October 29- November 1, **Tel –Aviv, Israel.**

Aviram M. "Dietary antioxidants and paraoxonase attenuate macrophage foam cell formation and atherosclerosis development" Nutrition, Lipids and Atherosclerosis. November 17-18, **Madrid, Spain.**

2005-

Aviram M. "Dietary tomatoe's lycopene reduces heart diseases" International Conference on Antioxidant and Lycopene, March 16, **London**.

Aviram M. "Macrophage NADPH oxidase and foam cell formation: Anti-atherosclerotic role for dietary antioxidants and for paraoxonase", Workshop on "new insights in mechanisms of vascular diseases", May 20-21, **Baveno, Maggiore Lake, Italy.**

Aviram M. "Oxidative stress and cardiovascular dieases: protective role of dietary antioxidants". Visual Function – Insights from the revolution in biology at the molecular level". June 14-16,

Tel Aviv, Israel.

Aviram M. and Fuhrman B. "Pomegranate and CVD: pomegranate juice polyphenolic antioxidants protect against oxidative stress and atherosclerosis development", Symposium on Human Health effect of Fruits and Vegetables, August 18-20, **Quebec City, Canada.**

2004-

Aviram M. "Paraoxonases protective mechanisms against oxidative stress, macrophage foam cell formation and atherosclerosis development". The 74th EAS Congress. April 17-20, **Seville, Spain** (Speaker and Organizer).

Aviram M. "Paraoxonase (PON1) protects against lipids peroxidation and attenuates atherosclerosis development". The First International Meeting on Paraoxonases – Basic and Clinical Directions of Current Research. April 23-24, **Ann Arbor, MI, USA** (Speaker and Organizer).

Aviram M. "HDL-associated paraoxonase1 (PON1) antioxidant and anti-inflammatory properties: PON1 protection against macrophage foam cell formation and atherosclerosis development". September 2-6, HDL Workshop. **Heraklion, Crete, Greece.**

Aviram M. "Paraoxonases and macrophage foam cells formation". Frontiers in Cardiovascular Science, October 14-17, **Eilat, Israel.**

Aviram M. "Paraoxonases and diabetes". The Russell Berrie 1st international Diabetes Symposium. October 17-19, **Jerusalem, Israel.**

Aviram M. "Dietary antioxidants and paraoxonases: protection against cardiovascular diseases". International Conference on Mechanisms of action of Nutraceuticals (ICMAN). November 12-14, **Waynsville, Haywood County, Western North Carolina, U.S.A.** (Speaker and Organizer).

2003-

Aviram M. "Mediterranean dietary antioxidants inhibit macrophage foam cell formation". September 20-23,**Cannes,France** (Speaker and Organizer).

Aviram M. "Molecular pharmacology of herbal medicine and botanical products in the treatment of vascular disease: the pomegranate example". Traditional herb medicines in Atherosclerosis Symposium. September 24-27, **Taipei, Taiwan.**

Aviram M. "Flavonoids-rich nutrients with potent antioxidant activity prevent atherosclerosis development". The XIIIth International Symposium on Atherosclerosis. September 28- October 2, **Kyoto, Japan** (Speaker and Organizer).

Aviram M. "Paraoxonases protects against oxidative stress and atherosclerosis progression at the humoral and cellular levels". Frontiers in Cardiovascular Science, October 23-26, **Eilat** (Speaker and Organizer).

2002-

Aviram M. "Oxysterols induce macrophage NADPH oxidase activation". XIth Biennial Meeting of the Society for Free Radical Research International: Role of free radicals, oxidants and antioxidants, in molecular and cell biology and life processes: New developments and techniques". July 16-20, **Paris, France.**

Aviram M. 1st Plenary Meeting, EC Concerted Action on Process for the Assessment of Scientific Support for Claims on Foods – PASSCLAIM. September 4-6, **Berlin, Germany**(Advisory Board member).

Aviram M. "Paraoxonase and Atherosclerosis". XIII Lipid-Meeting, Leipzig", September 30th – October 2nd, **Leipzig, Germany.**

Aviram M. "Paraoxonase protects against oxidative stress and atherosclerosis progression". Frontiers in Cardiovascular Science, October 3-6 , **Eilat**
(Speaker and Organizer).

Aviram M. "Oxidative stress, macrophage foam cell formation and Atherosclerosis". European Community Center of Excellence Workshop on: "Cardiovascular dysfunctions in hyperlipidemia and diabetes". October 9-13, **Bucharest, Romania.**

Aviram M. "Wine flavonoid antioxidant against LDL oxidation". Vinsalude 2002 Chile: Wine and Health International Congress, October 20-23, **Santiago, Chile** (Speaker and Organizer).

Aviram M. "Macrophage, Foam Cell formation and Atherosclerosis under Oxidative Stress: studies in the apolipoprotein E deficient mice". Biocenter Oulu Graduate School Advance Course: From cells to tissues: signaling and mechanisms,
December 3, **Oulu, Finland.**

2001-

Aviram M. "New Avenues in Atherosclerosis Research:Genomics and New Therapeutical Perspectives". March 14-15, **Montreal, Canada.**

Aviram M. "Wine polyphenols, LDL oxidation and atherosclerosis", "Conference on Wine and Alcohol in Health and Disease". April 26-29, **Palo Alto, California, USA.**

Aviram M. "Dietary antioxidants and cardiovascular diseases" International Conference on Mechanisms of action of Nutraceuticals (ICMAN). October 10-14, **Dubrovnik, Croatia.**

2000-

Aviram M. "Anti-atherogenicity of the licorice derived isoflavane glabiridin: inhibitory role in LDL oxidation and macrophage foam cell formation". The Oxygen Club of California, 2000 orld Congress, March 1-4, **Santa Barbara, California, USA.**

Aviram M. "Role of angiotensin II in lipoprotein oxidation and cholesterol metabolism in the vascular wall". The 15th American Society of Hypertension (ASH) meeting.
May 15-19, **New York, NY, USA.**

Aviram M. "Polyphenols inhibit LDL Oxidation and Atherosclerosis". XXth International Conference on Polyphenols, September 11-15, **Freising, Germany.**

Aviram M. "Antioxidant activity of statins as well as of pomegranate juice", PTBNM Polish society for atherosclerosis research MEM, October, 23-26 in **Krag Casttle, Koszalin, Poland.**

1999-

Aviram M. "Paraoxonase, LDL Oxidation and Macrophage Foam Cell Formation".
The 2nd Rappaport – Mayo Symposium on vascular biology: therapeutic horizons in cardiovascular disease. May 23-27, **Rochester, MN, USA.**

Aviram M. "Review of Human Studies related to vascular function". ILSI Europe Workshop on Markers of Oxidative Damage and Antioxidant protection. June, 28-30, **Prague, Czech Republic.**

Aviram M. "Functional Ingredients in Wine". 4th Karlsrushe Nutrition Symposium on Vegetables and Fruit for better Nutrition and Health: Scientific evidence and Practical Experiences. October, 10-12, **Karlsruhe, Germany.**

Aviram M. "Oxidized LDL and Atherosclerosis: Role of Antioxidants and Paraoxonase" 10th International Dresden Symposium on Lipoproteins and Atherosclerosis.
December, 9-11, **Dresden, Germany.**

1998-

Aviram M. "LDL oxidation in athpatients and the effect of drug therapy". The Pfizer Lecture, February 9, **Montreal, Canada**.

Aviram M. "LDL oxidation and atherosclerosis: antiatherogenicity of antioxidants". The first regional meeting on medical science-The roles of free radicals in health and disease"
March 22-27, **Jerusalem-Amman, Israel-Jordan** (Speaker and Organizer).

Aviram M. "Anti-atherogenicity of antioxidants against LDL oxidation" The Second International Conference on Natural Antioxidants and Anticarcinogens in Nutrition, Health and Disease (NAHD). June 24-27, **Helsinki, Finland.**

Aviram M. "Tomato's lycopene and β-carotene inhibit LDL oxidation. Nutracon, July 20-22, **San Antonio, Texas.**

Aviram M. "Paraoxonase reduces lipoprotein oxidation: a possible role for its per-like activity". The European Atherosclerosis Society, 70 EAS Meeting, September 6-9, **Geneva, Switzerland.**

Aviram M. "Human serum paraoxonase, lipoprotein oxidation and atherosclerosis". The 5th International Union of Biochemistry and Molecular Biology (IUBMB) Conference on the Biochemistry of Health and Diseases. October, 18-22, **Jerusalem, Israel.**

Aviram M. " Dietary antioxidants against LDL oxidation in cardiovascular diseases". American College for advancement in Medicine, November 20-22, **Phoenix, Arizona.**

1997-

Aviram M. "Macrophage-mediated oxidation of LDL" - The first joint meeting of the Rappaport Institute and the University of Dundee. January 26-28, **Dundee, Scotland, U.K.**

Aviram M. "LDL oxidation by macrophages". The first international workshop on molecular biology of mononuclear phagocyte differentiation and activation. **Advisory Board Expert.** May 2-3, **Regensburg, Germany.**

Aviram M. "Inhibition of LDL oxidation by macrophage - and by LDL - associated antioxidants" - The 68th meeting of the European Atherosclerosis Society (EAS): Molecular Cell Biology and Atherosclerosis. May 7-10, **Brugge, Belgium.**

Aviram M. "Antiatherogenicity of statin therapy in hyperchoelsterolemic patients: effects on platelet activation and on LDL oxidation". The 29th annual meeting of Japan Atherosclerosis Society (JAS). June 5-6, **Tokyo, Japan.**

Aviram M. "Dietary antioxidants and LDL oxidation". The 2nd world conference of the international society for molecular nutrition and therapy. August 2-4, **Winnipeg, Canada.**

Aviram M. "Lipoprotein oxidation and atherosclerosis"- **Chairman of the workshop.**
11th International Symposium on Atherosclerosis. October 5-9, **Paris, France.**

Aviram M. "Macrophage -mediated oxidation of LDL and atherosclerosis".
The First Rappaport-Mayo Symposium on Vascular Biology. December 1, **Haifa, Israel.**

1996-

Aviram M. "Role of oxidized LDL in the development of atherosclerosis". The Menarini Series on Cardiovascular Diseases: Advances in Cardiovascular Pathology. January 26-27, **Florence, Italy.**

Aviram M. "Antioxidants against LDL oxidation and Atherosclerosis". March 1st, **Naarden, The Netherlands.**

Aviram M. "Lycopene and Atherosclerosis". BioMed, Inaugural Lecture Program,
March 2nd, **Birmingham, U.K.**

Aviram M. "Interrelationship among platelet activation, LDL oxidation and foam cell formation in hypercholesterolemic patients: antiatherogenic effects of statin therapy". Asian-Pacific Congress on Vascular Diseases. March 11-15, **Singapore.**

Aviram M. "Macrophage relevance in the atherome plaque formation. Effects of pravastatin in the inhibition of cellular cholesterol synthesis and increase of LDL receptor activity in macrophages". International Symposium on Coronary Prevention and Lipidic Control: Fisiopathologic basis and new Therapeutic Consensus. Laboratories Dr. Esteve. S.A., April 19-20, **Barcelona, Spain.**

Aviram M. "Oxidative modification of LDL and Atherosclerosis". V Simposio International Sorbe, Alimentation, Lipidos Y Atherosclerosis. May 30- June 1, **Madrid, Spain.**

Aviram M. "Macrophage-mediated oxidation of LDL depends on the balance between cellular oxygenases and antioxidants". 66th Congress of the European Atherosclerosis Society, July 13-17, **Florence, Italy.**

Aviram M. "Interrelationship among platelet activation, LDL oxidation and foam cell formation in hypercholesterolemic patients: antiatherogenic effects of statin therapy" 66th Congress of the European Atherosclerosis Society, July 13-17, **Florence, Italy.**

Aviram M. "LDL enrichment with tomato's lycopene increases its resistance to oxidation in the atherosclerotic, apolipoprotein E deficient transgenic mice" 11th International symposium on Carotenoids. August 18-23, **Leiden, The Netherlands.**

Aviram M. "Macrophage-mediated oxidation of LDL: role of cellular-and lipoprotein-associated antioxidants". 2nd International Conference on Lipoprotein Oxidation and Atherosclerosis: Biological and Clinical Aspects. September, 12-14, **Pavia, Italy.**

Aviram M. "Inhibition of LDL oxidation by macrophage-and LDL-associated antioxidants". **Chairman of the Symposium**. International Symposium on vitamins and antiproliferative agents in prevention of atherosclerosis. The 4th Congress of the Polish Society for Atherosclerosis Research. October 3-6, **Zokopane, Poland**.

Aviram M. "Red wine quercetin inhibits LDL oxidation and aggregation in the atherosclerotic, apolipoprotein E deficient, transgenic mice". The First Workshop on Wine and Human Health. October 9-11, **Udine, Italy.**

Aviram M. "Macrophage proteoglycans contribute to the binding and uptake of oxidized low density lipoprotein (Ox-LDL). The 69th Scientific Sessions, American Heart Association (AHA), November 10-13, **New Orleans, Louisiana, U.S.A.**

1995-

Aviram M. "Macrophage uptake of oxidized LDL inhibits lysosomal sphingomyelinase, thus causing the accumulation of unesterified cholesterol". 5th Rappaport Symposium:

Modified Lipoproteins, Antioxidants and Atherosclerosis. **Chairman of the Symposium.** May, 8-12, **Shavei Zion, Israel.**

Aviram M. "Oxidative stress affects LDL-platelet interactions and induce foam cell formation". XVth Congress of the International Society on Thrombosis and Haemostasis. June 11-16, Jerusalem, **Israel.**

Aviram M. "Platelet activation, LDL oxidation and foam cell formation". 1st International Meeting on Interventional Cardiology. June 18-23, **Jerusalem, Israel.**

Aviram M. "Platelets and macrophage cholesterol accumulation". 11th IFCC European Congress of Clinical Chemistry. July 2-7, **Tampere, Finland.**

Aviram M. "Oxidized LDL and macrophage accumulation of unesterified cholesterol". Third Scientific Meeting of the Polish Society for Atherosclerosis Research. October 5-8, 1995, **Cracow, Poland.**

Aviram M. "Dietary Antioxidants against LDL oxidation and atherosclerosis". Annual Meeting of the Indian Society for Atherosclerosis Research. December 8-10. **New Delhi, India.**

Aviram M. "Inhibition of LDL oxidation by carotenoids: a comparative study of lycopene and β-carotene". International Conference of Food Factors. December, 10-15. **Hamamatsu, Japan.**

1994-

Aviram M. "Macrophage-mediated modification of LDL and atherosclerosis". IV International Symposium on Lipids and Atherosclerosis. May 5-7. **Madrid, Spain.**

Aviram M. "Oxidized LDL and Atherosclerosis". Senior Investigator Arcol Prize laureate. Hundred years celebration to Pasteur Institute. June 2-4. **Lille, France.**

Aviram M. "LDL lipid modifications increases its Atherogenicity". Second Congress of the Polish Society for Atherosclerosis Research. June 4-7. **Szczecin, Poland.**

Aviram M. "phospholipase-modified LDL and Atherogenesis". 8th International Dresden Lipid Symposium. June 10-12. **Dresden, Germany.**

Aviram M. "Macrophage-mediated Oxidation of LDL and Atherosclerosis". Society for Free Radical Research. September 16-18. **Pavia, Italy.**

1993-

Aviram M. "Dietary olive oil decrease LDL atherogenicity. The VII Creteil Symposium on Nutrition, Lipids and Lipoproteins". February 12. **Paris, France.**

Aviram M. "Beyond cholesterol: Modification of lipoproteins and increased atherogenicity". International symposium on Atherosclerosis, Inflammation and Thrombosis". March 21-24, **Florence, Italy**.

Aviram M. and Rosenblat M. "Macrophage-mediated oxidamodification of LDL". Joint Meeting of the German and Societies for Cell Biology (DGX and NVVC), March 28-31. **Munster, Germany**.

Aviram M. "LDL lipids modifications and increased atherogenicity". 62nd European atherosclerosis Society. September 5-9. **Jerusalem, Israel**.

Aviram M. "Antioxidant mediated inhibition of LDL-modifications reduces its atherogenicity". International SFRR symposium on Antioxidants, Inflammation, Cardiovascular and Ophthalmic disease. Servier Award. September 30 - October 2. **Valencia, Spain**.

1992-

Aviram M. "Serotonin increased macrophage uptake of oxidized low density lipoprotein". XI International Symposium on Drugs affecting Lipid Metabolism, May 13-16. **Florence, Italy**.

Aviram M. "Increased susceptibility to activation and increased uptake of low density lipoprotein by cholesloaded macrophages". 59 European Atherosclerosis Society Congress. May 17-21. **Nice, France.**

Aviram M. "Lipids and platelet function in the development of atherosclerosis in diabetes". The Second International Symposium on Diabetes and Atherosclerosis, September 5-7. **Carlsbad, Czechoslovakia**.

Aviram M. "Macrophage-mediated LDL oxidation required LDL binding to the LDL receptor". 33rd International conference on the Biochemistry of Lipids (ICBL). September 7-10. **Lyon, France**.

Aviram M. "Phospholipase D-modified low-density lipoprotein is taken up by macrophages at an increased rate: A possible role for phosphatidic acid". 65th Scientific Sessions, American Heart Association (AHA), November 16-19. **New Orleans, LA, U.S.A**.

1991-

Aviram M. "Platelet mediated cholesterol accumulation in macrophages". 9th International Symposium on Atherosclerosis. October 6-11. **Chicago, (Rosemont) IL, U.S.A**.

1990-

Aviram M. "Platelet enhancement of macrophage cholesterol accumulation: a novel mechanism

for atherogenesis". 2nd Mediterranean Congress of Angiology. September 16-22. **Antalya, Turkey**.

Aviram M. "Platelet enhancement of macrophage cholesterol accumulation: A novel mechanism for atherogenesis". European Lipoprotein Club Meeting. September 17-20. **Tutzing, Germany**.

Aviram M. "Platelet enhancement of macrophage cholesterol accumulation: A novel mechanism for atherogenesis". 63rd Scientific Sessions, American Heart Association. November 12, 1990, **Dallas, TX, U.S.A.**

1989-

Aviram M. "Lipase modified LDL and macrophage cholesterol accumulation". International Meeting on atherosclerosis (EAS). April 20-22. **Wien, Austria.**

Aviram M. "Macrophage uptake of oxidized LDL is increased by platelet secretoryproducts". European Lipoprotein Meeting September 11-14. **Tutzing, Germany**.

Aviram M. "Lovastatin inhibits LDL oxidation". International symposium on drugs affecting lipid metabolism. November 8-11. **Houston, TX, U.S.A.**

Aviram M. "Macrophage uptake of oxidized LDL is increased by platelet secretory products". 62nd Scientific Sessions of the American Heart Association. November 13-16. **New Orleans, LA, U.S.A.**

1988-

Aviram M. "Lipase-modified LDL results in cholesterol accumulation in macrophages". American Federation for Clinical Research (AFCR), Western Section. February 16-19. **Carmel, CA, U.S.A.**

Aviram M. "Low density lipoprotein triglyceride content determines its interaction with cells". 8th International Symposium on Atherosclerosis. October 9-13. **Rome, Italy**.

Aviram M. "Triglyceride content of LDL affects the interaction of apo B with cells". 61st Scientific Sessions, American Heart Association, November. **Washington, D.C., U.S.A.**

1987-

Aviram M. "Intralipid infusion abolishes the ability of human serum to cholesterol load cultured macrophages". 60th Scientific Sessions, American Heart Association. November 16-19. **Anaheim, CA, U.S.A.**

1985-

Aviram M. "Antithrombotic effect of plasma chylomicrons on endothelial cells: differences between dietary cream and cod liver oil". Seventh International Symposium on Atherosclerosis. October 6-10. **Melbourne, Australia**.

1984-

Aviram M. "Chylomicron atherogenicity: Plasma chylomicrons decrease platelet function". Seventh Annual Conference on Lipoproteins. The European Lipoprotein Club. September 10-13. **Tutzing, Germany**.

1980-

Aviram M. "Low-density lipoprotein receptors on human platelets". American Federation for Clinical Research (AFCR). February 10-15.**Washington D.C. U.S.A.**

PUBLICATIONS

Theses

1. M.Sc. thesis: "Energy requirement of the intracellular breakdown of ribonucleic acid".

2. D.Sc. thesis: "Turnover of tyrosine-aminotransferase in hepatoma tissue culture cells".

Refereed Papers in Professional Journals

1. Tal M., **M. Aviram**, A. Kanarek, and A. Weiss. Polyuridylic acid binding and translating byEscherichia coli ribosomes: stimulation by protein I, inhibition by aurintricarboxylic acid. ***Biochim Biophys Acta*** **281**: 381-392 (1972).

2. **Aviram M.,** and A. Hershko. Influence of ATP on the degradation of rapidly labeled RNA in cultured hepatoma cells. ***Biochem Biophys Res Commun*** **65**: 1303-1310 (1975).

3. **Aviram M.,** and A. Hershko. Interconversion of multiple forms of tyrosine-aminotransferase in vitro and in vivo in cultured hepatoma cells. ***Biochim Biophys Acta*** **498**: 83-90 (1977).

4. Rapoport J., **M. Aviram**, C. Chaimovitz, and J.G. Brook. Defective high-density lipoproteins composition in patients on chronic hemodialysis. A possible mechanism for accelerated atherosclerosis. ***N Engl J Med*** **299**:1326-1329 (1978).

5. Brook J.G., A. Lavy, **M. Aviram**, and O. Zinder. The concentration of high-density lipoprotein in patients with type IV hyperlipoproteinemia and the effect of clofibrate. ***Atherosclerosis*** **36**: 461-469 (1980).

6. **Aviram M.,** J.G. Brook, A.M. Lees, and R. S. Lees. Low density lipoprotein binding to human platelets: role of charge and of specific amino acids. ***Biochem Biophys Res Commun*** **99**: 308-318 (1981).

7. Brook J.G., **M. Aviram**, R. Luboshitzky and D. Barzilai. High density lipoprotein and arteriosclerosis. Different patterns in primary hypothyroidism and type IIa hyperlipoproteinemia. ***Isr J Med Sci*** **17**: 318-322 (1981).

8. **Aviram M.**, and J.G. Brook. The effect of human plasma on platelet function in familial hypercholesterolemia.***Thromb Res*** **26**: 101-109 (1982).

9. Brook J.G., **M. Aviram**, A. Viener, E. Shilansky, and W. Markiewicz. High-density lipoproteinsubfractions in normolipidemic patients with coronaratherosclerosis. ***Circulation*** **66**: 923-926 (1982).

10. **Aviram M.**, R. Luboshitzky, and J.G. Brook. Lipid and lipoprotein pattern in thyroid dysfunction and the effect of therapy. ***Clin Biochem*** **15**: 62-66 (1982).

11. **Aviram M.**,Z. Blumenfeld, J.G. Brook, A. Levy and J. Brandes. High-density lipoprotein and its subfractions in cord blood. ***Singapore J Obstet Gynecol*** **13**: 107-114 (1982).

12. **Aviram M.**, J.G. Brook, S. Mokady, R. Diukman, B. Fogel and U. Cogan. Changes in rabbits' plasma lipoprotein pattern induced by substituting soybean protein for dietary animal protein. ***Nutr Rep Intern*** **26**: 569-579 (1982).

13. Brook J.G., **M. Aviram**, M. Oettinger and M. Sharf. The effect of estrogen implants on high density lipoproteins and its subfractions in women in their early pre-mature menopause. ***Maturitas*** **4**: 257- 265 (1982).

14. Ron D., I. Oren, **M. Aviram** ,O.S.Better,and J.G. Brook. Accumulation of lipoprotein remnants particles in patients with chronic renal failure. ***Atherosclerosis*** **46**: 67-75 (1983).

15. **Aviram M.**, and J.G. Brook. Platelet interaction with high and low-density lipoproteins. ***Atherosclerosis*** **46**: 259-268 (1983).

16. Brook J.G., G. Winterstein, and **M. Aviram**. Platelet function and lipoprotein levels after plasma-exchange in patients with familial hypercholesterolemia. ***Clin Sci*** **64**: 637-642 (1983).

17. **Aviram M.** Plasma lipoprotein separation by discontinuous density gradient ultracentrifugation in hyperlipoproteinemic patients. ***Biochem Med*** **30**:111-118 (1983).

18. Blumenfeld Z., **M. Aviram,** J.G. Brook, and J.M. Brandes. Changes in lipoprotein and subfractions following oophorectomy and oestrogen replacement in perimenopausal women. ***Maturitas*** **5**:77-84 (1983).

19. **Aviram M.**, and J.G. Brook. The effect of blood constituents on platelet function: role of blood cells and plasma lipoproteins. ***Artery*** **11**: 297-305 (1983).

20. Ron D., **M. Aviram**, and J.G. Brook. High density lipoprotein in octogenarians. ***Biochem Med*** **30**:253-260 (1983).

21. Markel A., J.G. Brook, Y. Levy, **M. Aviram**, and M.B.H. Youdim. Increased platelet adhesion and aggregation in hypertensive patients: effect of atenolol. ***Br J Clin Pharmacol*** **16**:663-668 (1983).

22. **Aviram M.**, and J.G. Brook. Characterization of the effect of plasma lipoproteins on platelet function in vitro. ***Haemostasis*** **13**:344-350 (1983).

23. Mokady S., U.Cogan, G. Dreifus, **M. Aviram** and J.G. Brook. Plasma lipids and lipoproteins in normo-and hyperlipidemic rabbits fed plant protein diets. ***Nutr Res*** **4**:897-902 (1984).

24. Baruch Y., J.G. Brook, S. Eidelman, and **M. Aviram**. Increased concentration of high density lipoprotein plasma and decreased platelet aggregation in primary biliary cirrhosis. ***Atherosclerosis*** **53**:151- 162 (1984).

25. Viener A., J.G. Brook, and **M. Aviram**. Abnormal plasma lipoprotein composition in hypercholesterolemic patients induces platelet activation. ***Eur J Clin invest*** **14**: 207-213 (1984).

26. Shmulewitz A., J.G. Brook, and **M. Aviram**. Native and modified low-density-lipoprotein interaction with human platelets in normal and homozygous familial-hypercholesterolemic subjects. ***Biochem J*** **224**: 13-20 (1984).

27. **Aviram M.,** and J.G. Brook. Selective release from platelet granules induced by plasma lipoproteins. ***Biochem Med*** **32**: 30-33 (1984).

28. Levy Y., **M. Aviram**, G. Spira, I. Tatarsky, J.G. Brook, and A. Carter. Plasma cholesterol concentration and extra lipid band in monoclonal gammopathies. ***Postgrad Med J*** **60**:449-453 (1984).

29. Haim S., J.G. Brook, A. Gilhar, R. Friedman-Birnbaum, A. Markel, **M. Aviram,** A. Marmur, and M.B.H. Youdim. Platelet function in Behcet's disease. ***J Dermatol*** **11**:117-120 (1984).

30. Weschler A., **M. Aviram,** M. Levin, O.S. Better, and J.G. Brook. High dose of L-carnitine increases platelet aggregation and plasma triglyceride levels in uremic patients on hemodialysis. ***Nephron*** **38**: 120-124 (1984).

31. Berkovitch Y., A. Marmur, J.G. Brook, and **M. Aviram**. Platelet adhesion determination in whole blood using a simple stagnation flow method. ***Ann Biomed Eng*** **12**:335-346 (1984).

32. **Aviram M.**, B. Fuhrman, and J.G. Brook. Chylomicrons from patients with type V hyperlipoproteinemia inhibit platelet function. ***Atherosclerosis*** **56**: 157-167 (1985).

33. **Aviram M.,** G. Winterstein, and J.G. Brook. Differential effect of platelet inhibitors in normal and in hypercholesterolemic subjects. ***Br J Clin Pharmacol*** **19**: 715-719 (1985).

34. **Aviram M.,** R.J. Deckelbaum, and J.G. Brook. Platelet function in a case with abetalipoproteinemia. ***Atherosclerosis*** **57**:313-323 (1985).

35. Markel A., J.G. Brook, and **M. Aviram**. Increased plasma triglycerides, cholesterol and apolipoprotein E during prolonged fasting in normal subjects. ***Postgrad Med J*** **61**:395-400 (1985).

36. Marmur A., E. Braunstein, J.G. Brook, and **M. Aviram**. Sedimentation and adhesion of blood platelets under centrifugal force. ***J Colloid Interface Sci*** **104**: 390-397 (1985).

37. **Aviram M.**, Y. Schecter, and J.G. Brook. Chylomicron-like particles in severe hypertriglyceridemia. ***Lipids*** **20**: 211-215 (1985).

38. **Aviram M.**, C.R. Sirtori, S. Colli, P. Maderna, G. Morazzoni, and E. Tremoli. Plasma lipoproteins affect platelet malondialdehyde and thromboxane B2 production. ***Biochem Med*** **34**:29-36 (1985).

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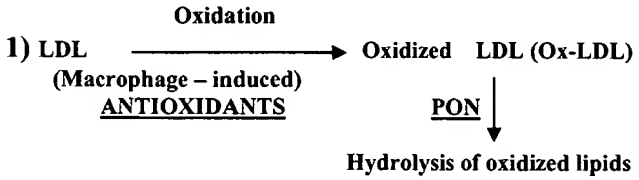
Appendix

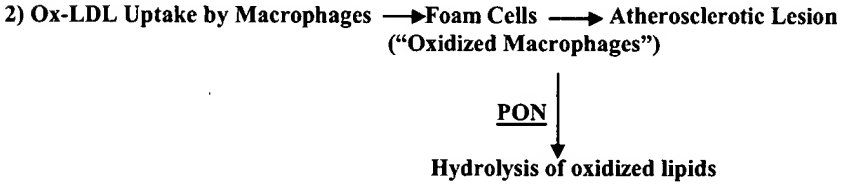
Research Accomplishments

Michael Aviram

Broad Objective

Macrophage Cholesterol accumulation and Foam Cell formation under Oxidative Stress during Atherogenesis: Role of Dietary Antioxidants and of Paraoxonase.





Specific Research Areas

1. Lipoproteins Oxidation and Atherosclerosis (1980 – present)

Oxidative stress is thought to play a key role in the development of atherosclerosis, the major cause of morbidity and mortality in the western world. We have shown that LDL oxidation, as well as additional **lipoprotein modifications** contribute to enhanced atherogenicity of LDL (1-5). Studies on mechanisms of **oxidized LDL (Ox-LDL) retention to extracellular matrix (ECM) proteoglycans** (heparan and chondroitin sulfate), as related to the lipoprotein uptake by macrophages scavenger receptors, leading to cellular accumulation of cholesterol and oxysterols, are carried out in our laboratory (6-9). **Macrophage – mediated oxidation of LDL** and foam cell formation is the hallmark of early atherogenesis, and we have demonstrated the role of cellular oxygenases (such as NADPH oxidase) and of antioxidants (such as the glutathione

system) in macrophages, as well as in LDL oxidation. We have demonstrated that under oxidative stress, not only the
lipoproteins are oxidized, but also the cellular lipids. We showed the presence of Ox-LDL and of **lipid peroxidized macrophages** in the atherosclerotic lesion, and demonstrated that these oxidized cells are able to oxidize LDL even in the absence of transition metal ions (10-12). We have shown **increased LDL oxidation in patients** with increased risk for atherosclerosis (hypercholesterolemia, hypertension, diabetes, chronic renal failure). Drug therapy in these patients (hypocholesterolemic "statins", ACE inhibitors) reduced the patient's increased LDL oxidation (13-14).

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2. Dietary Antioxidants and Atherosclerosis (1990 – present)

Dietary antioxidants that inhibit LDL oxidation can attenuate atherosclerosis development , and we have demonstrated indeed such properties for vitamin E, carotenoids (lycopene, β-carotene), and mainly for polyphenolic flavonoids, such as those found in pomegranate (hydrolyzable tannins), wine (flavonols), licorice (isoflavans) , ginger (flavonones) and olive oil (phenolics).

We have provided evidence that the inhibitory effect of some flavonoids on LDL oxidation (and on atherosclerosis) is related to their interaction with the lipoprotein directly, as well as to their accumulation in arterial macrophages and inhibition of cellular oxygenases (1-9).

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3. Paraoxonase ,Lipids Peroxidation and Atherosclerosis (1997 – present)

Under excess oxidative stress, antioxidants capability to block the formation of Ox-LDL may not be sufficient. We have recently demonstrated that HDL - associated **Paraoxonase** (PON1) can hydrolyze oxidized lipids (such as cholesteryl linoleate hydroperoxides) in oxidized lipoproteins and in atherosclerotic lesion, and thus may act as a second line of defense against oxidative stress. Combination of potent antioxidants (flavonoids) together with paraoxonase was shown in our laboratory to attenuate atherogenesis, secondary to reduced oxidative stress and reduce macrophage uptake of oxidized lipoproteins via the scavenger receptors (1-14).

Representative Publications :

1. **Aviram M.**, Rosenblat M., Bisgaier C.L., Newton R.S., Primo-Parmo S.L., and La Du B.N.: J. Clin. Invest. 101:1581-1590 (1998).
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:			
	Luba COHEN		
Serial No.:	09/955,933		
Filed:	September 20, 2001	Group Art Unit:	1651
For:	LICORICE EXTRACT FOR USE AS A MEDICAMENT		
		Attorney Docket:	37229
Examiner:	Deborah K. Ware		

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF PROF VAYA UNDER 37 CFR 1.132

1. I, Prof. Jacob Vaya am a senior researcher at Migal Galilee Technology Center since 1985. I have been investigating the chemical components of different plant extracts for the last 22 years. My CV is attached to this declaration.

2. Before giving this declaration I carefully read the article by Fuhrman et al., published at Am. J. Clin. Nutr. 1997; 66:267-75, of which I am a co-author, (hereinafter Fuhrman) and U.S. patent No. 6,280,776 to Sha et al. (hereinafter "Sha").

3. Fuhrman describes lowering the susceptibility of Low density lipoprotein (LDL) to oxidation by administering a water-insoluble licorice extract. LDL is the major cholesterol carrier in the blood.

In re Application of: Luba Cohen
Serial No.: 09/955,933
Filed: September 20, 2001
Office Action Mailing Date: October 13, 2006

Examiner: Deborah K. Ware
Group Art Unit:
Attorney Docket: 37229

4. Fuhrman describes that lowering LDL susceptibility to oxidation occurs similarly by administrating glabridin, the major active constituent of the licorice water insoluble extract (Fuhrman extract).

5. Fuhrman describes that supplementation of either water insoluble licorice extract or its major active constituent glabridin, results in reduction in the atherosclerotic lesion area of mice aortic arch.

6. Fuhrman concludes that the above beneficial effect of the water insoluble licorice extract is due to its glabridin content.

7. Glabridin is a practically water insoluble compound and it can be extracted with high efficiency from the pulp which remains after water extract of the root. (Starting with water extraction of the root which removes all the water soluble components, and subsequently performing a second extraction using organic solvent which extracts the non-polar constituents remain in the pulp containing mainly glabridin). It is therefore unlikely to find glabridin in a water extract as that used by Sha.

8. Sha describes improving blood cholesterol level, blood sugar, and liver functions as achievable by taking a food supplement that contains up to 15% water-soluble licorice extract.

9. I refer herein to the licorice extract used by Sha as Sha's licorice extract, and to the conditions that Sha describes as being treated by a food supplement that contains Sha's extract – as Sha's conditions.

10. I refer herein to the licorice extract used by Fuhrman as Fuhrman's extract.

11. I was asked if common sense could lead me to replace Sha's extract with Fuhrman's extract in order to treat Sha's conditions. My answer is absolutely not. In the following I explain why this is so.

12. First of all, Sha explains that the main active ingredient in licorice is glyzyrrhizinic acid. Fuhrman describes her extract to be free of glyzyrrhizinic acid. It is against common sense in my field to take constituent X of a successful composition, and replace it with a constituent Y, that is free of the main active ingredient in X. There is actually no sense in doing so.

13. In accordance with Fuhrman, the main active agent in the Fuhrman extract is glabridin. Fuhrman describes that in *in vitro* and *in vivo* experiments, glabridin alone performed similar beneficial effects as did Fuhrman extract. In experiment I carried out with water-soluble extract of licorice, which I believe to be similar to that obtained by Sha, I found that glabridin is practically absent from the water-soluble licorice extract.

14. In summary, laboratory tests I have run showed that the constituent which is reported by Sha to be the active ingredient in Sha's extract is practically absent from Fuhrman's extract, and the component reported by Fuhrman to be the active ingredient in Fuhrman's extract is practically absent from Sha's extract.

15. Additionally, Fuhrman's extract and Sha's extract are different not only in content of glyzyrrhizinic acid, but in nearly all their constituents. Therefore, there is no reason to believe that the effect of one of them will be similar to the effect of the other, even if glyzyrrhizinic acid is irrelevant to the efficacy of Sha's extract.

16. It is clear to me that the two extracts are very different chemically based on the differences in their water solubility. In detail:

a. Sha's extract is water soluble. This means that most of it constituents are water-soluble substances.

b. Fuhrman's extract is water insoluble. This means that it has very little, if any, water-soluble substances.

c. From a. and b. above, it is straightforward that the two extracts are very different in their chemical constituents.

17. I was asked would one of ordinary skill in my field be motivated to replace Sha's water soluble extract with Fuhrman's water insoluble extract in order to improve shelf-life of the product. My answer is absolutely not, for the reasons detailed below:

a. From my experience I know that shelf-life of plant extracts is not correlated with their water solubility. Therefore, one of ordinary skill in my field would not expect lengthening the shelf-life of a product by replacing a water soluble ingredient with a water insoluble one, unless there is specific information suggesting that the replacement at issue would indeed lengthen the shelf-life. I don't know of the existence of such specific information.

b. I never heard on shelf-life problems associated with water soluble licorice extracts.

c. Even if one component has a longer shelf-life than another, a person of ordinary skill would not suggest exchanging between the two if they have as vastly different chemical constitution as the extracts of Sha and Fuhrman have.

18. Finally, I would like to comment that Sha's extract is very well known in my field, and has been used in folklore medicine for centuries. It is commercially available and being important component in the tobacco industry, and may be marketed without posting any unexpected regulatory problems. Fuhrman's extract, on

the other hand, is not so well known, and the regulatory authorities may be expected to post heavier requirements before allowing its marketing. Therefore, I believe that market forces join the other factors I explained above in challenging the desirability of the suggested replacement.

19. In conclusion, I believe that the idea of replacing Sha's extract with Fuhrman's extract is against common sense.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United states Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Vaya Jacob

2 January, 2008
Date

Prof. Jacob Vaya

Encl.:
Curriculum Vitae

CURRICULUM VITAE

1. Personal Details:

Date of birth:	October 1946
Country of birth:	Iraq
Identity No.:	74158056
Nationality:	Israeli
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2. Higher Education

1968-1971: BSc, Hebrew University of Jerusalem, Faculty of Chemistry, study in Chemistry and Biochemistry.

1971-1973: MSc, Hebrew University of Jerusalem, Faculty of Chemistry, Supervisor: Prof. Albert Zilkha; Thesis: "Sulfonation of Polyamides".

1974-1978: PhD, Weizman Institute of Science, Rehovot, Faculty of Chemistry, Supervisor: Prof. Mario D. Bachi; Thesis: "Investigations on β-Lactams Structurally Related to Penicillins and Cephalosporins". Major accomplishment - Total synthesis of (±)spirocyclopentanodinorpenicillin, synthesis of novel types of compounds: the thiomalonimides.

3. Additional Education, Training and professional certificates

1981-1985: Group Manager; **Teva** Pharmaceutical Industries. Research activities: isolation, purification and structure determination of new compounds isolated from bacteria. Project manager in development and improvement of processes for the production of different types of drugs (**attached list of internal publications**). Optimization of parameters, active involvement in pilot plan for production of drugs.

1985-1990: Team-Head; Migal - Galilee Technological Center.

Research activities: Development of processes and facilities for the production of essential oils and oleoresins from natural sources, optimization of processes from laboratory scale to pilot and eventually commercialization. Development of analytical methods for the identification and quantification of compounds from natural raw materials. Major accomplishment - Setting up commercial enterprise (owned by Israel Chemicals, Ltd.) for production of extract from natural raw materials (roots, seeds, fruits, plants, algae etc.). Our group was responsible for all the R&D and the QC activities to the enterprise.

Processes developed under the above activities:

a. Establishment of efficient installation for the production of essential oils in several tons of raw materials for batch.

b. Extraction and isolation of pigments (carotenoids) from paprika fruit as industrial process (achieving $1x10^6$ c.u.).

c. Development of process for the isolation of β-carotene from dry or paste algae (more than 80% β-carotene in the extract).

d. Development of efficient process for the selective removal of pesticides from ginseng extract (an industrial process).

e. Development efficient processes for the isolation of Partenolide from feverfew, eleutherosides B and E from eleutherococcus, flavonoides from licorice.

f. Development formulations from oleoresins for specific use (solubility, synergism, etc.).

g. Identification and testing of natural antioxidants for therapeutic uses. The effects of natural antioxidants on LDL (the main carrier of cholesterol in human plasma). Development of methods for early identification of atherosclerosis and markers for the study of oxidative stress. Correlation between oxysterols (oxidized cholesterol derivatives) and atherosclerosis development. Studying for correlation between structure of flavonoids and their ability to prevent LDL from oxidation (SAR). Studies on new natural

compounds, their isolation, structure elucidation and their *in vitro* and *in vivo* estrogen-like activity (phytoestrogens). The relation between the structures of phytoestrogens isolated from plants to their activities as agonist/antagonist to estradiol using computerized modeling (Insight, CeriousII). Developing markers for early diagnosis of oxidation damage in humans.

h. Isolation of natural whitening agents. The design and synthesis of new tyrosinase inhibitors.

i. Designing and synthesis of exogenous markers for the characterization of oxidative stress.

4. Appointments at academic institutions

1978-1981: Research Associate with Prof. T. Ross Kelly, Department of Chemistry, Boston College, Boston, Mass. USA. Field of research: Total synthesis of natural compounds. Major accomplishment - An efficient regiospecific total synthesis of Daunomycinone.

1998 (June-Oct). Partial sabbatical, Dept. Molecular Cell Biology at UC Berkeley. Studying interactions between flavonoids and proteins. Developed analytical methods for quantification of Pycnogenol. Established cooperation between our group at Migal and Prof. L. Packer group at Berkeley.

2001- Partial Sabbatical – Research Associate with Profs. A. Sevanian and E. Cadenas. School of Pharmacy, University of Southern California. The effect of phytoestrogens, in vivo models.

2005- Partial Sabbatical – , Centre for Neurotranslational Research, McGill University and LDI for Medical Research, Jewish General Hospital – Prof. Hyman Schipper. Establishing collaboration between the two groups investigating HO-1 and Alzheimer disease.

2003 – Received - Associate Professor

5. Administrative positions in academic institutions

2002-2005 – Head of the Dept. Biotechnology and Environmental Science, School of Science and Technology, Tel Hai Academic College.

6. Positions in non academic & research Institutions

1981-1985: Group Manager; Teva Pharmaceutical Industries (Israel).

1985-1990: R&D manager; Migal - Galilee Technological Center. Establishing factory producing flavors and fragrances – Galilee Aroma LTD.

7. Membership in professional organizations

1990- The Israeli Society of Oxygen and Free Radicals research

1995- The International Society of Free Radicals Biology and Medicine

1996- The European Atherosclerosis Society

1997- The Israeli Atherosclerosis Society

8. Research Grants

Date	Funding agency	Research topics	Grant - US $
1998-1999	Ministry of industry and commerce, Chief science	Phytoestrogens	240,000
2000-2001	Ministry of industry and commerce, Chief science	SAR in phytoestrogens	55,000
1998-2001	Ministry of Science-	Construction of bank of natural extracts.	75,000
2000-2003	Israeli Science Foundation	Agonist and antagonist to estradiol. SAR	135,000
2001-2002	Ministry of industry and commerce, Chief science	New Whitening agents	100,000
20001-2003	Ministry of Science Culture & Sport division for Agricultural and Environment	Development of inhibitors for tyrosinase	80,000
2004-2007	Israeli Science Foundation	The search for natural substrates of Paraoxonase	65,000
2004-2005	D-Cure	The effect of PON1 on early advances glycation products	54,000
2006	Ministry of industry and commerce, Chief science	Masking of protein surfaces for decreased antigenicity	130,000
2007	Ministry of industry and commerce, Chief science	Masking of protein surfaces for Increasing antigenicity	130,000

9. Teaching at Academic institutions

1990-1991: Tel Hai College, Upper Galilee, Israel. Course: Essential oils and extracts in the flavors and fragrances industry.

1992-1993: Tel Hai College, Upper Galilee, Israel. Courses: Antioxidants, chemistry, production and uses in the food and cosmetic industry.

1993-2005: Department of Biotechnology and Environmental Science, School of Science and Technology (B.Sc.), Tel Hai Academic College, Upper Galilee, Israel. Course: Organic Chemistry.

2003-2005: Department of Biotechnology and Environmental Science, School of Science and Technology (B.Sc.), Tel Hai Academic College, Upper Galilee, Israel. Course: Free Radicals and Oxidative Stress.

10. Supervision of Graduate Students

1990-1992. Aviv Cohen, "Preparation of Black pepper emulsion", M.Sc., Hebrew University of Jerusalem, School for Science and Technology, Cassali Institute for Applied Chemistry, with Prof. N.Garti and A. Aserin; Completed.

1991-1993. Vered Dangur, "Palladium Catalysis Elimination reactions", M.Sc. Faculty of Chemistry, Technion, Haifa, with Prof. E.Keinan; Completed.

1995-1998. Paula Belinky, "Effect of antioxidant constituents from the roots of *Glycyrrhiza glabra* (Licorice) on the oxidation of low density lipoprotein (LDL)", Ph.D. School of Medicine, Technion, Haifa, with Prof. M.Aviram; Completed.

1997-1999. Ayelet Nir, " Influence of stress on antioxidants defense system in apple; extraction and separation of components, and examination of their control of superficial scald". M Sc. Hebrew University of Jerusalem, Faculty of Pharmacy, with Dr. Roni Cohen, Prof. Ruth Ben Arye and Amos Levin. Completed.

2001-2006. Ohad Nerya, "Prevention of browning in plant tissues with new natural and synthetic inhibitors of tyrosinase". Ph.D. student, Hebrew University of Jerusalem, Dept. of Fruit Storage Research, with Prof. Ruth Ben Arye. Completed

2001-2005. Andrea Shochtman, " Characterization of oxidative stress processes and their products by chemical synthesized markers". Ph.D. student. School of Medicine, Technion, Haifa, with Prof. Michael Aviram. Completed

2006- Yuval Aluf - MSc. student. School of Medicine, Technion, Haifa, with Prof. John Finberg.

2006- Hagi Tabory- Ph.D. student. School of Medicine, Technion, Haifa, with Prof. Michael Aviram.

11. List of Publications:

11.1. Master and Doctoral Dissertation

M.Sc. – Sulfonation of polyamides. The Hebrew University, Jerusalem. Supervisor: Prof. Albert Zilkha.

Ph.D. Investigations on β-Lactams Structurally Related to Penicillins and Cephalosporins. The Weizman Institute of Science, Rehovot, Israel. Supervisor: Prof. Mario D. Bachi;

11.2. Books (Academic)

1. **Vaya J.** Packer L. 999. Entry on "Antioxidants" in McGraw-Hill Encyclopedia of Science & Technology, 9th Ed, Volume 2. Appearing in 5 languages, as a multi-media CD-ROM and in an on-line version.

2. Jacob Vaya, Snait Tamir, Dalia Somjen, Estrogen-Like Activity of Licorice Root Extract and its Constituents . Oxidative Stress and Disease **(2004),** 14 (Herbal and Traditional Medicine), 615-634. Editors; Lester Packer, Choon Nam Ong and Barry Halliwell, Publisher- Marcel Dekker Inc.

3. Michael Aviram. **Jacob Vaya** and Bianca Fuhrman. Licorice root flavonoid antioxidants reduce LDL oxidation and attenuate cardiovascular diseases. Oxidative Stress and Disease **(2004),** 14(Herbal and Traditional Medicine), 595-614. Editors; Lester Packer, Choon Nam Ong and Barry Halliwell, Publisher- Marcel Dekker Inc.

4. N.P. Seeram, Y. Zhang, Jess D. Reed, Christian G. Krueger, Erika Salas and **Jacob Vaya.** Phytochemical Constituents of Pomegranate (*Punica granatum* L.). **2006.**

5. Jacob Vaya. Novel designed probes for the characterization of oxidative stress in biological fluids, cells and tissues. Advanced Protocols in Oxidative Stress **(2008),** Editor; Donald Armstrong. Humana press, Totowa , New Jersey.

11.3 Articles in refereed journals

1. **Vaya J.,** Zilkha A. 1974. Sulfonation of polyamides. *Isr. J. Chem.* **12**: 873-878

2. Bachi M.D., **Vaya J.** 1976. Azetidin-2-oxo-4-thiones: Novel thermolytic product of β-lactam sulfoxides. *J. Am. Chem. Soc.* 98: 7825-7826

3. Bachi M.D., **Vaya J.** 1977. Reactions and properties of azetidin-2-oxo -4-thiones. *Tetrahedron Letters*, 2209-2212.

4. Bachi M.D., Frydman N., Sasson S., Stern C., **Vaya J.** 1997. Synthesis of (±)-penicillin and (±)-2-spirocyclopentanobisnorpenicillin systems. *Tetrahedron Letters*, 641-644.

5. Bachi M.D., **Vaya J.** 1979. Phosphinimines as useful intermediates in the synthesis of 3-(acylamino)-β-lactams. *J. Org. Chem.* **44**: 4393-4396.

6. Bachi M.D., Goldberg O., Gross A., **Vaya J.** 1980. Synthesis of 4-thioxo-2-azetidinones. *J. Org. Chem.* **45**: 1477-1485.

7. Bachi M.D., Goldberg O., Gross A., **Vaya J.** 1980. Properties and reactions of 4-thioxo-2-azetidinones. *J. Org. Chem.* **45**: 1481-.

8. bachi M.D., Sasson S., **Vaya J.** 1980. Studies related to penicillins and cephalosporins, part 6. Synthesis of the (±)dinorpenicillin-spirocyclopentane system. *J. Chem. Soc. Perkin I:* 2228-2232.

9. Kelly T.R., **Vaya J.,** Ananthasubramanian L. 1980. An efficient, regiospecific synthesis of (±)-daunomycinone. *J. Am. Chem. Soc.* **102:** 5983-5984.

10. Kelly T.R., Behforouz M., Echavarren A., **Vaya J.** 1983. Synthesis of the Rifamycin chromophore. *Tetrahedron Letter,* 2331-2334.

11. Kelly T.R., Ananthasubramanian L., Borah K., Gillard W.J., Goerner N.R., King P.F., Lyding M.J., Tsang W.G., **Vaya J.** An efficient regiospecific synthesis of (±)-daunomycinone. *Tetrahedron.* 40: 4569-4577.

12. Keinan E., Kumar S., Dangur V., **Vaya J.** 1994. Evidence for a cyclic mechanism in (η^3-allyl) palladium chemistry. Promotion of β-hydride elimination by unsaturated organometallics. *J. Am. Chem. Soc.* **116**: 11151.

13. Masaphy S., Levanon D., **Vaya J.**, Henis Y. 1993. Isolation and characterization of a novel Atrazine metabolite produced by the fungus Pleurotus Pulmonarius, -chloro-4-ethylamino-6-(1-hydroxyisopropyl)amino-1,3,5-triazine. *Appl. Environ. Microbiol.* 59: 4342-4346.

14. Degani G., Gal E., **Vaya J.** 1994. In vitro biosynthesis of steroids in ovary of asymchronic Trichogaster trichopterus (Pallus 1770). *Comp. Biochem. Physiol.* 109B: 715-723.

15. Vaya J., Belinky P., Aviram M. 1997. Antioxidant constituents from licorice roots: isolation, structure elucidation and antioxidative capacity toward LDL oxidation. *Free Radic. Biol. Med.* 23: 302-313.

16. Fuhrman B., Buch S., **Vaya J.**, Belinky P., Coleman R., Hayek T., Aviram M. 1997. Licorice alcoholic extract and its major polyphenol glabridin protect LDL against lipid peroxidation: in vitro and ex-vivo studies in human and in the atherosclerotic apolipoprotein E deficient mice. *Amer. J. Clin. Nutr.* 66, 267-275.

17. Hayek, T., Fuhrman, B., **Vaya, J.**, Rosenblat, M., Belinky, P. A., Coleman, R., Elis, A. and Aviram, M., 1997. Reduced progression of atherosclerosis in the apolipoprotein E deficient mice following consumption of red wine, or its polyphenols quercetin or catechin, is associated with reduced susceptibility of LDL to oxidation and to aggregation, *Arterioscler. Thromb. Vasc. Biol.* 17, 2744-2752.

18. Belinky P., Aviram M., **Vaya J.** 1998. The antioxidative effects of the isolated isoflavan Glabridin on endogenous constituents of LDL during its oxidation. *Atherosclerosis.* 137, 49-61.

19. Belinky P., Aviram M., Saeed Mahmood., **Vaya J.** 1998. Structural aspects of the inhibitory effect of glabridin on LDL oxidation. *Free Radic. Biol. Med.* 24, 1419-1429.

20. Rosenblat, M.; Belinky, P. A.; **Vaya, J.**; Levy, R.; Merchav, S.; Aviram, M. Macrophage enrichment with the isoflavan glabridin inhibits NADPH oxidase-induced cell-mediated oxidation of low density lipoprotein. A possible role for protein kinase C. *J. Biol. Chem. 1999 May 14;274(20):13790-9*

21. Hadi Moini, Antonio Arroyo, **Vaya, J.**; Lester Packer. 1999. Bioflavonoid effects on the mitochondrial respiratory electron transport chain and cytochrome c redox state. *Redox Report.* 4, *35-41.*

22. Fuhrman Bianca., **Vaya J.**, Belinky Paula., Aviram Michael. **1999**. The isoflavan glabridin inhibits LDL oxidation : structure and mechnistic aspects. *Spec. Publ.-R. Soc. Chem.* 240 (Natural antioxidants and anticarcinogens in nutrition, health and disease), 161-165.

23. Irit Maor., Marielle Kaplan, Tony Hayek., **Vaya. J.**, Aaron Hoffman and Michael Avirm. Oxidized monocyte-derived macrophages in aortic atherosclerotic lesion from apolipoprotein E-deficient mice and from human carotid artery contain lipid Peroxides and Oxysterols. *Biochem Biophys Res Commun* **2000**;269(3):775-780

24. Michael Aviram, Emiliya Hardak, **Vaya, J.**, Saeed Mahmood, Simcha Milo, Aaron Hoffman, Scott Billicke, Dragomir Draganov and Mira Rosenblat. Human serum paraoxonases (PNO1), Q and R selectively decrease lipid peroxides in human coronary and carotid atherosclerotic lesions: PON1 esterase and peroxidase-like activities. *Circulation.* **2000** May 30;101:2510-2517.

25. Snait Tamir, Mark Eizenberg, Dalia Somjen., Naftali Stern, Rayah Shelach, Alvin Kaye, and **Vaya, J.**, Estrogenic and Antiproliferative Properties of Glabridin from Licorice in Human Breast Cancer Cells. *Cancer Research*. **2000**, 60, 5704-5709.

26. Vaya, J., Saeed Mahmood, Tony Hayek, Ehud Grenadir, Simcha Milo, Aaron Hoffman, and Michael Aviram. Selective distribution of oxysterols in human plasma, plasma lipoproteins and atherosclerotic lesions. *Free Radical Research.* **2001**; 34, 485-497.

27. Kaye AM, Spatz M, Waisman A, Sasson S, Tamir S, **Vaya J.**, Somjen D. Paradoxical interactions among estrogen receptors, estrogens and SERMS: mutual annihilation and synergy. *J Steroid Biochem Mol Biol.* **2001**; 76(1-5):85-93.

28. Aviram Michael and **Vaya J.**, Markers for low-density lipoprotein oxidation. *Methods Enzymol.* **2001**;335:244-256.

29. Snait Tamir, Mark Eizenberg, Dalia Somjen, Sarit Izrael and **Vaya, J.**, Estrogen Like-Activity of Glabrene and other Constituents Isolated from Licorice Root. *J Steroid Biochem Mol Biol.* **2001.** 78:291-298.

30. Vaya, J., and Michael Aviram. Nutritional Antioxidants: Mechanisms of Action and Medical Applications. *Current Medicinal Chemistry-Immunology, Endocrinology & Metabolic Agents.* **2001**. 1:99-117

31. Kaplan M, Hayek T, Raz A, Coleman R, Dornfeld L, **Vaya J.**, Aviram M. Pomegranate juice supplementation to atherosclerotic mice reduces macrophage lipid peroxidation, cellular cholesterol accumulation and development of atherosclerosis. *J. Nutr.* **2001.** 131:2082-2089.

32. Snait Tamir, Sarit Izrael, **Vaya, J.**, The Effect of Oxidative Stress on ERα and ERβ Expression. *The Journal of Steroid Biochemistry and Molecular Biology* .September **2002**. 81: No 4-5, ;327-332.

33. Vaya, J., Somjen, D.; Tamir, S. The role of the isoflavan's 2' hydroxyl in diverse biological activities. Editor(s): Pasquier, Catherine. Proceedings of [the] Biennial Meeting of the Society for Free Radical Research International, 11th, Paris, France, July 16-20, 2002 (2002), 567-574. Publisher: Monduzzi Editore, Bologna, Italy

34. Vaya, J., Saeed Mahmood., Amiram Goldblum., Michael Aviram, Nina Volkova, Amin Shaalan., Ramadan Musa, Snait Tamir. Inhibition of LDL oxidation by flavonoids in relation to their structure and calculated enthalpy. *Phytochemistry*. **2003.** 62 (1): 89-99.

35. Ohad Nerya, **Vaya, J.,** Ramadan Musa, Sarit Izrael, Ruth Ben-Arie and Snait Tamir.. Glabrene and Isoliquiritigenin as Tyrosinase Inhibitors. *Journal of Agricultural and Food Chemistry*. 51(5): 1201-1207, **(2003)**.

36. Rivka Ofir, Snait Tamir, Soliman Khatib, **Vaya, J.,** Inhibition of serotonin re-uptake by licorice constituents. *The Journal of Molecular Neuroscience*, **(2003)**; 20:35-140.

37. Andrea Szuchman, Michael Aviram, Snait Tamir, **Vaya, J.**, The effects of oxidative stress on cholesterol, linoleic acid or tyrosine vs. their mixture. **(2003).** *Free Radical Research*. 37:1277-88.

38. Vaya, J., and Snait Tamir. The relation between the chemical structure of phytoestrogens and their estrogen-like activities **(2004)**. *Current Medicinal Chemistry*. 11:1333-1343.

39. Ohad Nerya, Ramadan Musa, Soliman Khatib, Snait Tamir, **Vaya, J.,** Chalcones as potent tyrosinase inhibitors. The effect of hydroxyl- positions and numbers **(2004)**. *Phytochemistry*, 65(10):1389-1395.

40. Somjen Dalia, Knoll Esther, **Vaya, J.,** Stern Naftali, Tamir Snait. Estrogen-Like Activity of Licorice Root Constituents Glabridin and Glabrene in Vascular Tissues *In Vitro* and *In Vivo*. **(2004).** *The Journal of Steroid. Biochemistry and Molecular Biology*. 91(3):147-155.

41. Dalia Somjen, Sara Katzburg, **Vaya, J.**, Alvin M. Kaye, D. Hendel, G.H. Posner, Snait Tamir. Estrogenic activity of glabridin and glabrene from

licorice roots on human osteoblasts and prepubertal rat skeletal tissues. **(2004).** *The Journal of Steroid Biochemistry and Molecular Biology*. 91 (4-5), 241-246.

42. Tzchori, Itai; Degani, Gad; Elisha, Ronit; Eliyahu, Rivka; Hurvitz, Avshalom; **Vaya, J.**,; Moav, Boaz. The influence of phytoestrogens and oestradiol-17β on growth and sex determination in the European eel (Anguilla anguilla). *Aquaculture Research.* **(2004),** 35(13), 1213-1219.

43. Soliman Khatib, Ohad Nerya, Ramadan Musa, Snait Tamir, **Vaya, J.**, Chalcones as potent tyrosinase inhibitors. The importance of a 2,4-substituted resorcinol moiety. *Biorganic & Medicinal Chemistry.* **(2005)**. 13(2), 433-441.

44. Rosenblat M, **Vaya, J.,** Shih D, Aviram M. Paraoxonase 1 (PON1) enhances HDL-mediated macrophage cholesterol efflux via the ABCA1 transporter in association with increased HDL binding to the cells: a possible role for lysophosphatidylcholine. **(2005)**. Atherosclerosis. 2005 Mar;179(1):69-77.

45. Nerya O., Ben-Arie R., Dannai O., Tamir S. and **Vaya, J.** Inhibition of mushroom browning. **(2005)**. Acta Hort. 682:1885-1888.

46. Szuchman A, Aviram M, Soliman K, Tamir S, **Vaya, J.**, Exogenous N-linoleoyl tyrosine marker as a tool for the characterization of cellular oxidative stress in macrophages. **(2006).** *Free Radic Res.* Jan;40(1):41-52.

47. Ohad Nerya, Ruth Ben-Arie, Tal Lussato, Ramadan Musa, Soliman Khatib and **Vaya, J.,** Prevention of *Agaricus bisporus* postharvest browning with tyrosinase inhibitors. **(2006)**. *Postharvest Biology and Technology*. 39(3): 272-277,

48. Mira Rosenblat, Leonid Gaidukov, Olga Khersonsky, **Vaya, J.,** Roni Oren, Dan S. Tawfik, and Michael Aviram. The catalytic histidine dyad Of HDL-associated serum paraoxonase 1 (PON1) is essential for PON1 - mediated inhibition of LDL oxidation and stimulation of macrophage cholesterol efflux. **(2006)**. *J. Biol. Chem.* 281(11), 7657-7665,

49. **Vaya, J.**, Saeed Mahmood. Flavonoid content in leaf extracts of the fig (*Ficus carica* L.), carob (*Ceratonia siliqua* L.) and pistachio (*Pistacia lentiscus* L.). **(2006),** *BioFactors.* 28(3-4), 169-175.

50. **Vaya, J.,** Wei Song, Soliman Khatib, Guoyan Geng, and Hyman M. Schipper. Effects of Heme Oxygenase-1 Expression on Sterol Homeostasis in Rat Astroglia. **(2007).** *Free Radicals Biology & Medicine*. 42, 864-871.

51. Soliman Khatib, Ramadan Musa, **Vaya, J.,** Linoleoyl tyrosine 2'-deoxyguanosyl ester as a novel designed synthetic probe for the characterization of oxidative stress. **(2007).** *Bioorganic Medicinal Chemistry*. 15 (11), 3661-66.

52. Soliman Khatib, Ohad Nerya, Ramadan Musa, Snait Tamir, Tal Peter and **Jacob Vaya,** Enhanced 2,4-substituted resorcinol hydrophobicity augments tyrosinase inhibition potency – a study relating structure to activity. **(2007).** *J. Medicinal Chemistry.* 50 (11). 2676-2681.

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Vaya J., Cohen Z., VanDen Brouke E., Schonberger C. March 1984. Preparation of aminoglutethimide. Teva.

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Vaya J., Barak G., Hirsch D. Nov. 1994. Preparation of pyrilamine maleate, part III. Optimization of the procedure for the preparation of pyrilamine base. Teva.

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12. Participation in Scientific Conferences (During the last 6 years)

Year	Subject	Name of conference/year
2000	Poster/ Human serum paraoxonases	Society for research, prevention and treatment of atherosclerosis/Eilat
2000	Poster/Flavonoids as inhibitors of LDL oxidation/ SAR study	Symposium/ Oxidative stress and atherosclerosis/ Oslo-Norway
2000	Poster/Oxysterols as marker for atherosclerosis development	XIIth international symposium on atherosclerosis/ Stockholm, Sweden
2001	Inhibition of LDL oxidation	II International Symposium on Natural antioxidants –Beijing -China
2001	Poster/The affect of oxidative stress on Estrogen receptors Era and Erb	8th Annual Meeting of the Oxygen Society- Durham, North Carolina - USA
2002	International Atherosclerosis Society	Paris

2003	Free radicals	Ionina- Greece
2004	Lecture/Prevention and treatment of Atherosclerosis	Eilat- Israel
2004	Lecture/ Chalcones as potent whitening agents- SAR study.	XXII International Conference on Polyphenols, Helsinki , Finland
2004	Lecture/ Exogenous tyrosine linoleate marker as a tool for characterization of cellular OS.	Jerusalem, Israel.
2005	Lecture -Marker for Oxidative stress	International Analytical Chemistry Conference. Tel Aviv
2005	Lecture- Marker for oxidative stress Lecture- Marker for Oxidative stress	McGill University, Faculty of nutrition, Montreal McGill University, LDI, The Jewish General Hospital
2006	Lecture -Effects of Heme Oxygenase-1 Expression on Sterol Homeostasis in Rat Astroglia	The 22nd Annual Meeting of the Israel Society for Oxygen and Free Radical Research. Tel Aviv

13. Current research

1. Oxidative stress and diseases –

1.1- : Multifunctional substrate for early detection of oxidative stress susceptibility and application to **Parkinson's disease**. This study utilizes an innovative molecule developed by us to characterize oxidative stress fingerprint of biological tissues. The novel marker constructed from multifunctional molecule of linoleic acid/tyrosine/guanosine (LTG) used to study oxidative/nitrative fingerprint for peripheral blood of Parkinsonian patients by comparison to normal controls, and by comparison to preclinical models of oxidative stress. The research program include experiments designed: a) To establish the oxidative stress profile for LTG in vitro using dopaminergic cell lines and a variety of stressors. b) To establish the oxidative stress profile for LTG in vivo using a rat model of increased dopaminergic turnover (unilateral 6-hydroxydopamine nigral lesion). c) To determine the oxidation/nitration of LTG by patient and control plasma, using blood from volunteer controls and patients of both sexes and of different ages. The controls are chosen to balance the patient group in age and sex. d) Results in patients are also related to severity of symptoms as determined by standard neurological criteria.

Collaborators: Prof. John Finberg, Faculty of Medicine, Technion, Haifa.

1.2. Heme oxygenase-1/sterol interaction in **Alzheimer disease-** Excess brain cholesterol can be eliminated via the ABC1A transporter and HDL cholesterol efflux pathways, and by esterification, oxidation to 24-, 25- or 27-hydroxy cholesterol (oxysterols) or conversion to bioactive steroids. Collaborative data from our

laboratories in Canada (H. M. Schipper) and Israel (J. Vaya) indicate that HO-1 over-expression in astroglia profoundly influences cholesterol and oxysterol metabolism in these cells. **Aims**: Building on our initial data set, experiments have been designed to test the following **hypotheses**: **#1-** HO-1 up-regulation in cultured astroglia suppresses cellular cholesterol levels by inhibiting the cholesterol biosynthetic pathway and/or augmenting cholesterol efflux via LXR activation. **#2-** Decreased cholesterol and cholesterol precursor concentrations and increased oxysterol levels correlate with augmented HO-1 protein expression in AD-affected human brain tissue.

Collaborators: Prof. Hyman Schipper- McGill University, Centre for Neurotranslational Research, Jewish General Hospital, Quebec, Canada.

2. **Method for obtaining modified proteins and viruses with intact native binding site. The aim.** The present study relates to methods for obtaining modified proteins, e.g. antibodies, and viruses with an intact native binding site and decreased antigenicity. **The principle idea**: There are several techniques for binding compounds to proteins in order to change proteins properties obtaining (a) reduction of protein immunogenicity, (b) change of the protein's surface properties and (c) increase of the plasma half-life when a protein is supplemented as drug. In most of these methods polymers are used for the conjugation of which two major polymers are the polyethylene glycol and Dextran or Dextran derivatives. The major disadvantage of these coating techniques is the fact that they can't distinguish between the different domains of the protein and bind non-specifically to protein, therefore reduce proteins' activity. However, in this research program we **aim** to overcome this obstacle in a simple and elegant procedure in which proteins and viruses antigenicity is reduced without damaging the native active/binding site of the protein.

Collaborators: Prof. Jacob Pitcovski – Migal Galilee Technology Center.

3. Mutual associations between **Paraoxonase-1 (PON1)** and exogenous lactones. Atherosclerosis is associated with increased oxidative. Oxidized low-density lipoprotein (Ox-LDL), as well as oxidized high-density lipoprotein (Ox – HDL), possesses atherogenic properties. Ox-LDL is taken up by macrophages at enhanced rate, whereas Ox-HDL losses its ability to induce cholesterol efflux from

macrophages, leading to accumulation of cholesterol and formation of foam cells, the hallmark of early atherosclerosis. PON1 in the serum is a HDL-associated enzyme which most of the anti-atherogenic properties of the HDL are thought to relate to PON1 activities, though, PON1 substrate is not yet known. **Aims:** To investigate the effects of PON1 on exogenous substrate such as unique lactonic drugs and nutrients, as well as the effect of such lactonic substances on PON1 structure and activity. Upon incubation of PON1 with exogenous lactones analyses is performed of PON1 anti-atherogenicity, alteration in PON1/substrate structure changes in oxido/redox status and related enzymes and PON1 glutathionization.

Collaborators: Prof. Michael Aviram, School of Medicine, Technion, Haifa.

14. Referees

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3. Prof. Lester Packer. Dept. Molecular Cell Biology, Univ. California at Berkeley, Berkeley, CA, 94720. Tel: 510-642-1872. Fax: 510-642-8313.

4. Professor Hyman M. Schipper, MD, PhD, Department of Neurology and Medicine McGill University. Director, Centre for Neurotranslational Research. Lady Davis Institute for Medical Research. Montreal, QC, Canada. Ph: 514-340-8260. FAX:514-340-7502. hyman.schipper@mcgill.ca

Endothelium-mediated Coronary Blood Flow Modulation in Humans

Effects of Age, Atherosclerosis, Hypercholesterolemia, and Hypertension

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Abstract

The effects of age, atherosclerosis, hypertension, and hypercholesterolemia on vascular function of the coronary circulation were studied by subselective intracoronary infusions of acetylcholine, which releases endothelium-derived relaxing factor, and papaverine, which directly relaxes vascular smooth muscle, in normal patients ($n = 18$; no risk factors for coronary artery disease), in patients with evidence of early atherosclerosis but normal cholesterol levels and normal blood pressure ($n = 12$), in patients with hypertension without left ventricular hypertrophy ($n = 12$), and in patients with hypercholesterolemia ($n = 20$). Papaverine-induced maximal increases in coronary blood flow were significantly greater in normals, but no differences were noted between the groups of patients with early atherosclerosis, with hypertension, and with hypercholesterolemia. The capacity of the coronary system to increase blood flow in response to acetylcholine was similar in normal and normocholesterolemic patients with epicardial atherosclerosis and/or hypertension but was significantly impaired in patients with hypercholesterolemia, irrespective of evidence of epicardial atherosclerotic lesions. Age ($r = -0.62$, $P < 0.0001$) and total serum cholesterol levels ($r = -0.70$; $P < 0.0001$) were the only significant independent predictors of a blunted coronary blood flow response to acetylcholine. Thus, hypercholesterolemia and advanced age selectively impair endothelium-mediated relaxation of the coronary microvasculature in response to acetylcholine, whereas endothelial dysfunction is restricted to epicardial arteries in age-matched normocholesterolemic patients with evidence of coronary atherosclerosis and/or hypertension. (*J. Clin. Invest.* 1993. 92:652–662.) Key words: endothelium-derived relaxing factor • coronary artery disease • acetylcholine • coronary vasomotor tone • risk factors

Introduction

The endothelium covers the inner surface of all blood vessels and has been recognized as playing a major role in modulating vascular smooth muscle tone by synthesizing and metabolizing vasoactive substances (1), including an endothelium-derived relaxing factor (EDRF)[1] (2), which is released after the stimulation of muscarinic receptors on endothelial cells by acetylcholine as well as by other agonists or physical stimuli (3, 4). Produced by endothelial cells, this factor traverses the subendothelial space and activates smooth muscle cell guanylate cyclase to increase cyclic guanosine monophosphate levels, leading to smooth muscle relaxation (5, 6). Evidence has been provided that nitric oxide, derived from L-arginine, or a related compound, may account for the biological activity of EDRF (7, 8).

Recently, it has become apparent that atherosclerosis markedly impairs this important function of the endothelium in large conduit vessels from a variety of experimental animals (9–11) as well as in humans (12–14). The observation that endothelium-dependent vasodilation of conduit vessels is abnormal in the presence of atherosclerosis is not surprising, because the thickened intima associated with atherosclerosis may act as a barrier to vasoactive substances released from the endothelium as well as because progression of atherosclerosis leads to an injury of the endothelium itself (15). Indeed, we have recently demonstrated a hierarchical structure in the impairment of endothelium-dependent responses with progressive disease in human epicardial conductance vessels in vivo (16).

Although possibly important in the genesis of vascular spasm, this abnormality of conduit vessel function probably contributes little to the regulation of coronary blood flow in the absence of spasm, because myocardial perfusion is regulated predominantly by microvessels < 200 μm in diameter (17). Although a functional abnormality of the coronary microvasculature has been recognized as a cause of chest pain in patients with angiographically normal coronary arteries and angina (18), little is known about the endothelium-mediated regulation of the coronary microvasculature in humans. Unlike the walls of larger arteries, the walls of resistance vessels do not develop overt atheroma after exposure to high levels of cholesterol (19). Nevertheless, several recent experimental studies did show that endothelium-dependent relaxation is abnormal in the resistance vessels of cholesterol-fed atherosclerotic animals (20–22). These results suggest that, despite the heterogeneity in the vascular susceptibility to develop overt atheroma in response to the atherogenic substance, the exposure of blood vessels to increased levels of circulating cholesterol induces abnormalities in vascular function in both conductance and resistance vessels. Yet, early in the course of coronary atherosclerotic disease before the development of hemodynamically significant epicardial artery lesions, an altered endothelial regulation of blood vessels would be of substantial pathophysiological importance if it would occur at flow-regulating sites within the coronary circulation. In addition, the presence of

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1. *Abbreviations used in this paper:* CAD, coronary artery disease; EDRF, endothelium-derived relaxing factor.

conditions contributing to the development of coronary atherosclerosis, such as hypertension and advanced age, may independently affect endothelial function of the coronary microvasculature and thereby exacerbate the pathophysiological consequences of coronary artery disease.

Thus, the present study was designed to assess the responsiveness of the coronary microvasculature in patients with early stages of epicardial atherosclerosis without flow-limiting stenoses; to evaluate the effects of plasma cholesterol levels on endothelium-mediated coronary blood flow modulation; to determine the effects of atherosclerosis associated with arterial hypertension on endothelium-mediated vascular function of the coronary circulation; and to evaluate whether age independently affects vascular function at the level of the coronary microvasculature.

Methods

Patient population

The study population included 62 patients undergoing routine diagnostic cardiac catheterization. These patients were classified into four groups on the basis of their history and the presence or absence of atherosclerosis on the diagnostic coronary angiogram. Patients with unstable angina, recent myocardial infarction, a clinical history suggestive of variant angina, valvular heart disease, clinical evidence of heart failure, and diabetes mellitus were excluded. In addition, no patient had angiographic or echocardiographic evidence of left ventricular hypertrophy. The epicardial artery vasomotor responses of 22 of these patients have been previously reported (16). Written informed consent was obtained from all patients before the study. The study protocol was approved by the Ethical Committee of the University of Freiburg.

Normal control group. The normal control group comprised 18 patients (6 women, 12 men). Normalcy was defined as: absence of a history of arterial hypertension (defined as chronically elevated blood pressure ≥ 150/95 mmHg) or hypercholesterolemia (total serum cholesterol level within the 75th percentile adjusted for age and sex); angiographically normal, smooth coronary arteries without luminal irregularities and a vasodilator response of the left anterior descending coronary artery (the territory under study) to the intracoronary infusion of acetylcholine up to a dosage of 7.2 μg/min; no evidence of segmental wall motion abnormalities and a left ventricular ejection fraction > 55% assessed by biplane cineventriculography; and a normal coronary flow reserve exceeding 4.0 in response to 7 mg papaverine subselectively infused into the left anterior descending coronary artery.

The mean age of these patients was 48.2±9.3 yr, ranging from 22 to 60 yr. Mean total serum cholesterol level was 200.9±21.3 mg%, ranging from 176 to 241 mg% at the time of the study. 16 patients underwent diagnostic coronary angiography for evaluation of atypical chest pain, and intermittent left bundle branch block was the cause for referral in two patients.

Coronary artery disease (CAD) group. 12 patients had angiographic evidence of coronary atherosclerosis, but < 30% luminal narrowing of the left anterior descending artery. Of these patients, four had > 50% luminal narrowing of either the right coronary artery or the left circumflex artery. The left anterior descending artery was angiographically normal in five patients, and demonstrated luminal irregularities with < 30% luminal narrowing in seven patients. All patients had normal total serum cholesterol levels ranging from 176 to 234 mg% (mean 209.8±17.6 mg%). None of these patients had a history of arterial hypertension requiring the initiation of antihypertensive therapy by the primary physician. Their mean age was 50.2±6.7 yr, ranging from 38 to 60 yr. Two patients were women and 10 were men.

Hypertension group. 12 patients had a history of arterial hypertension requiring the initiation of antihypertensive therapy by the primary physician. None of these patients had angiographic or echocardiographic evidence of left ventricular hypertrophy. In seven of these patients, the left anterior descending artery appeared angiographically normal, whereas luminal irregularities were visible in five patients. All patients had normal total serum cholesterol levels ranging from 179 to 232 mg% (mean 212.8±21.3 mg%). Their mean age was 55.9±6.5 yr, ranging from 47 to 68 yr, and three were women.

Hypercholesterolemia group. 20 patients had total serum cholesterol levels exceeding the 75th percentile adjusted for age and sex. The cholesterol levels ranged from 246 to 346 mg% (mean 284.2±30 mg%). 13 patients had an angiographically normal appearing left anterior descending artery, whereas luminal irregularities were visible in seven patients. Their mean age was 52.7±11.7 yr, ranging from 22 to 68 yr. 10 of these patients had a history of arterial hypertension, but no patient had angiographic or echocardiographic evidence of left ventricular hypertrophy. 3 patients were women and 17 were men. None of the patients in this study had a history of myocardial infarction in the territory of the left anterior descending coronary artery. All patients demonstrated a normal left ventricular contraction pattern in the anterior and septal left ventricular wall and a normal global ejection fraction as assessed by biplane cineventriculography. Left ventricular end-diastolic pressure was ≤ 13 mmHg in all patients.

Study protocol

Vasoactive medications, including calcium channel blockers, angiotensin-converting enzyme inhibitors, and long-acting nitrates, were withheld ≥ 24 hours before cardiac catheterization. No patient received beta-adrenergic blockers within 48 h before the study. A total of eight patients were on aspirin therapy during the study (one in the normal, three in the CAD, two in the hypercholesterolemia, and two in the hypertension group). Diagnostic left heart catheterization and coronary angiography were performed by a standard percutaneous femoral approach. After completion of the diagnostic catheterization, an additional 5,000 U of heparin were given intravenously and an 8 F guiding catheter (Schneider, Zurich, Switzerland) was introduced into the left main coronary artery. A 3 F catheter (Monorail-Doppler; Schneider) with a 20 MHz pulsed Doppler crystal was advanced into the left anterior descending artery via a 0.014-in guide wire. The Doppler catheter was carefully positioned to obtain a stable flow velocity signal. Before introducing the Doppler catheter into the guiding catheter, the flow velocity recordings were referenced to zero and calibrated.

After stable baseline conditions were obtained, acetylcholine was selectively infused into the left anterior descending artery via the Doppler catheter to assess endothelium-dependent increases in coronary blood flow. Increasing dosages of acetylcholine (0.036, 0.36, and 3.6 μg/ml) were infused at an infusion rate of 2 ml/min, lasting 3 min for each concentration. The lowest dose of 0.036 μg acetylcholine/ml corresponds to an estimated blood concentration in the coronary bed of 10^{-6} M, assuming a blood flow of 80 ml/min. Stepwise acetylcholine infusions were terminated either when vessel occlusion occurred or when the largest dose (3.6 μg/ml) was reached.

10 min after acetylcholine infusion, 7 mg papaverine was subselectively injected into the left anterior descending artery via the Doppler catheter to assess endothelium-independent coronary flow reserve in the territory of the left anterior descending artery. Previous studies (2) have demonstrated that the dose of 7 mg papaverine, subselectively infused into the left anterior descending artery, elicits a maximal increase in coronary blood flow without affecting global hemodynamic parameters.

Throughout the study, phasic and mean intracoronary blood flow velocity, heart rate, and aortic pressure (via the guiding catheter) were continuously measured. Serial hand injections of nonionic contrast material (Ultravist; Schering AG, Berlin, FRG) were performed during control, at the end of each acetylcholine infusion period, at recontrol after acetylcholine infusion, and after subselective infusion of papaverine.

Quantitative coronary angiography

The method of quantitative coronary angiography has been previously described (16, 23, 24). In brief, using a simultaneous biplane multid

rectional isocentric x-ray system (Siemens Bicor, Erlangen, FRG), the coronary arteries under study were positioned near the isocenter, biplane cine-angiograms were recorded at a frame rate of 25 frames/s, and enddiastolic cine frames were videodigitized and stored in the image analysis system (Mipron I; Kontron Electronics, Eching, FRG) in a 512 × 512 matrix with an 8-bit gray scale. Using the 12-cm field of view, the resulting pixel density was 7.3 pixels/mm. Automatic contour detection was performed by a previously described and validated method using a geometric edge differentiation technique (23, 24), and the exact radiological magnification factor of the measured segment was calculated to scale the data from pixels to millimeters (25). The accuracy and precision of this technique, as well as the reproducibility of serial measurements under routine clinical conditions, have been established in previous studies (23, 24).

Quantitative angiography of the epicardial artery was performed for two purposes. First, to determine cross-sectional area of the artery immediately distal to the radiopaque tip of the Doppler catheter to convert the Doppler-derived flow velocity to an estimate of coronary arterial flow. Second, to exclude limitations of coronary artery flow due to epicardial coronary artery constriction in response to acetylcholine by measuring the most constricting epicardial artery segment distal to the tip of the Doppler catheter, as previously suggested by Treasure et al. (26). To determine cross-sectional area of the artery, a 5–7-mm segment was measured immediately distal to the tip of the Doppler catheter. A series of diameter measurements was obtained for each scanline for the length of the arterial segment, displayed in graph form, showing diameter versus segment length, and the mean diameter value was calculated. Whenever possible, measurements were performed in both views of the biplane images using the radiopaque tip of the Doppler catheter for identification of corresponding vessel segments, and the vessels' cross-sectional area was calculated from both views assuming an elliptical shape. Only single-plane analysis was performed for those coronary segments demonstrating overlapping with other parts of the coronary tree in one view. In those cases (19 of 62 patients = 31%), vessel cross-sectional area was calculated assuming a circular shape. Measurement of the most constricting artery segment was performed in a similar fashion. However, instead of calculating the mean diameter value, the minimal absolute diameter of the analyzed segment was identified in both views and minimal cross-sectional area was calculated. Flow-limiting constriction was defined as > 50% cross-sectional area reduction compared with preacetylcholine cross-sectional area of the identical segment.

Data analysis

For estimation of directional changes in coronary blood flow, a coronary flow index was calculated by multiplying the mean Doppler-derived blood flow velocity with the computed cross-sectional area of the vessel segment immediately distal to the tip of the Doppler catheter. Since the injection of contrast material into the coronary circulation resulted in the typical biphasic response of coronary blood flow velocity with an initial decrease followed by an increase in flow velocity due to the hyperemic effects of the contrast material, the mean blood flow velocity immediately before the contrast injection was used for estimation of coronary blood flow. Blood flow responses to intracoronary acetylcholine infusion were analyzed in two ways and yielded similar results. First, as previously described (26), the slope of the dose–response relation to acetylcholine was calculated to correct for the fact that severe vasoconstriction with > 50% cross-sectional area reduction of the most constricting segment precluded the assessment of acetylcholine-induced increases in coronary blood flow. Using linear regression, the slope of the acetylcholine dose–response relation (% change in coronary blood flow index/dosage of acetylcholine) was calculated from the available doses for each patient. In the dose range of acetylcholine used in this study, a linear relation occurred between acetylcholine dose and the percent change in coronary blood flow index in each individual patient. The mean correlation coefficient was 0.91±0.07, ranging from 0.68 to 0.99, indicating good fit for the calculated regression lines used for the slope calculation. Second, because all patients received the 0.36 μg/ml dose of acetylcholine and no patient demonstrated > 50% constriction of the most constricting epicardial artery segment at this dose, the analysis was performed with the response to the single 0.36 μg/ml dose of acetylcholine.

Statistical analysis

All data are expressed as mean±SD, unless otherwise stated. Statistical comparisons were made by analysis of variance followed by the Student–Newman–Keul test. Linear-regression analysis was used to compare blood flow responses with age and serum cholesterol levels. Multivariate analysis using multiple stepwise regression techniques was performed to examine potential interactions between age, gender, cholesterol level, the presence or absence of angiographically visible epicardial artery atherosclerosis, and hypertension upon acetylcholine-induced blood flow responses. Statistical significance was assumed if a null hypothesis could be rejected at the 0.05 probability level.

Results

Baseline hemodynamic characteristics. The baseline systemic and coronary hemodynamic characteristics are summarized in Table I for the four groups of patients. There was no significant difference in heart rate, baseline epicardial artery cross-sectional area, or coronary blood flow index between the four groups of patients. As expected as a result of the enrollment criteria, mean aortic pressure was significantly higher in the group of patients with a history of hypertension.

Responses to acetylcholine and papaverine. No significant changes in mean aortic pressure or heart rate occurred during subselective infusion of either acetylcholine or papaverine. Fig. 1 illustrates the epicardial artery vasomotor response to the various interventions in a patient with hypercholesterolemia. In the normal control group, epicardial artery cross-sectional area increased during the acetylcholine infusion from 10.6±7.3 mm^2 at baseline to 12.8±7.9 mm^2 at the 3.6 μg/ml dose of acetylcholine corresponding to a 23.2±15.0% increase in lu-

Table I. Basal Systemic and Coronary Hemodynamic Characteristics

	Normals $n = 18$	CAD $n = 12$	Hypertension $n = 12$	Hypercholesterolemia $n = 20$
Heart rate (min^{-1})	71.7±9.7	73.8±9.9	69.8±10.3	70.9±9.6
MAP (mmHg)	90.1±5.3	89.1±8.4	101.2±7.0*	93.2±7.9
Epicardial artery luminal area (mm^2)	10.6±7.3	9.0±4.9	6.3±2.8	7.0±4.0
Coronary blood flow index (kHz × mm^2)	70.3±55.3	43.7±27.2	68.3±66.0	49.3±27.9

Values are mean±SD. MAP, mean aortic pressure; coronary blood flow index = Doppler-derived mean flow velocity × epicardial artery luminal area. * $P < 0.05$ versus normals, CAD, and hypercholesterolemia groups.

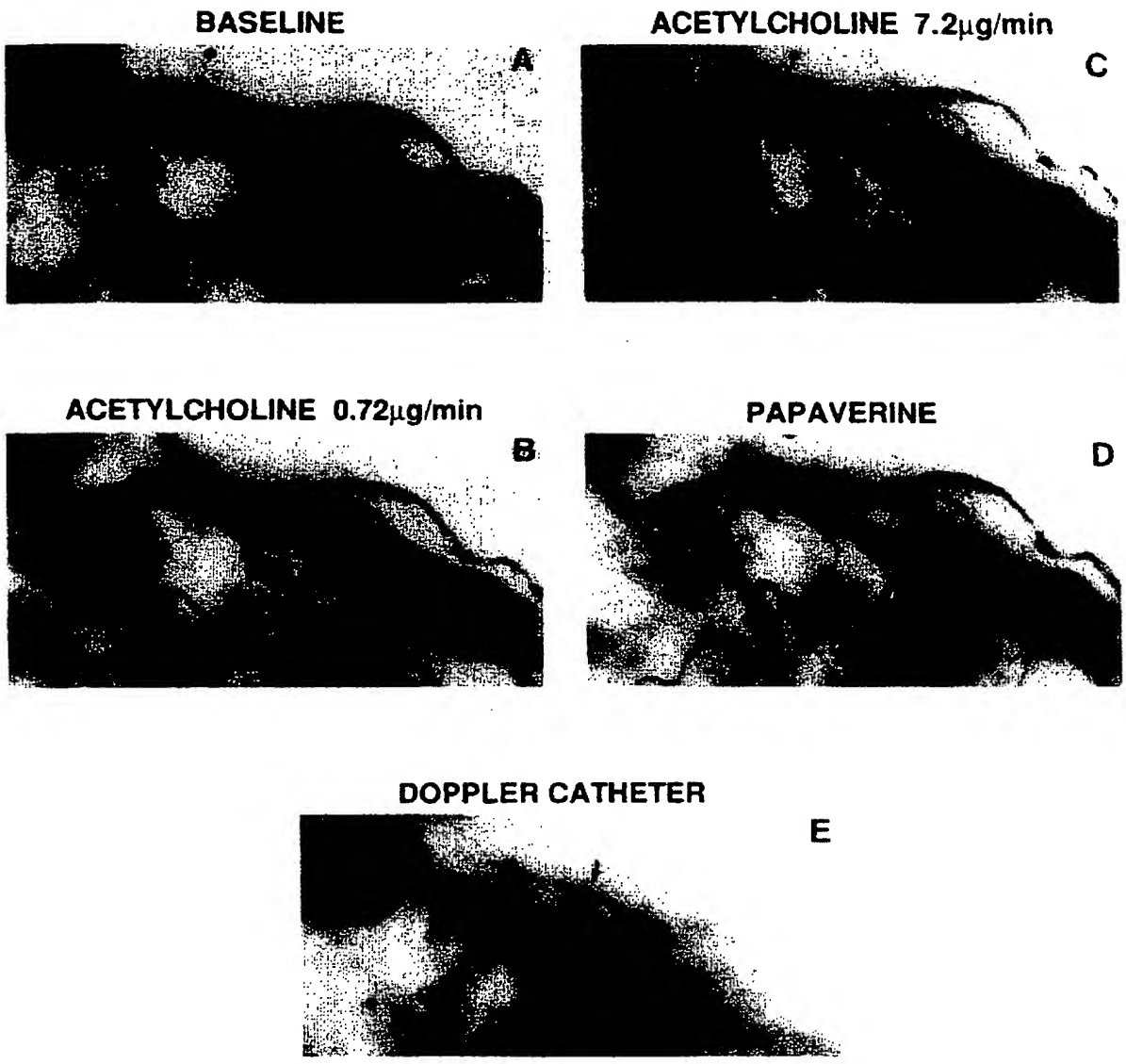

Figure 1. Coronary angiogram of a patient with hypercholesterolemia at baseline (*A; white arrows* indicate left anterior descending coronary artery distal to the Doppler infusion catheter), during 0.72 μg/min acetylcholine infusion (*B*), during 7.2 μg/min acetylcholine infusion (*C*), immediately after papaverine infusion (*D*), and position of the contrast-filled Doppler catheter (*small arrow, E*).

minal area (Fig. 2 *A*). In contrast, acetylcholine elicited a dose-dependent vasoconstrictor response of epicardial conductance vessels in patients with atherosclerosis, hypertension, and hypercholesterolemia (Fig. 2 *A*). Mean epicardial artery cross-sectional area decreased from 9.0±4.9 mm^2 at baseline to 6.7±3.6 mm^2 at the 3.6 μg/ml acetylcholine dose in patients with atherosclerosis, from 6.3±2.8 to 4.6±2.1 mm^2 in patients with hypertension, and from 7.0±4.0 to 4.9±3.9 mm^2 in patients with hypercholesterolemia. The extent of epicardial artery vasoconstriction was similar in all three groups of patients at each individual concentration of acetylcholine (Fig. 2 *A*). Moreover, the vasoconstrictor response did not significantly differ in angiographically normal arteries and arteries with a giographically visible wall irregularities.

Fig. 3 illustrates the intracoronary flow velocity tracin corresponding to the angiograms shown in Fig. 1 at the vario interventions. As shown in Fig. 2 *B*, the increases in corona blood flow indexes in response to acetylcholine were signi cantly different in the four groups of patients. Compared wi the patients with normal total serum cholesterol levels (norm control group, CAD group, and hypertension group), the vas dilator response of the coronary microcirculation to acetylch line was blunted in patients with hypercholesterolemia. Tl blunted coronary blood flow response achieved statistical sign

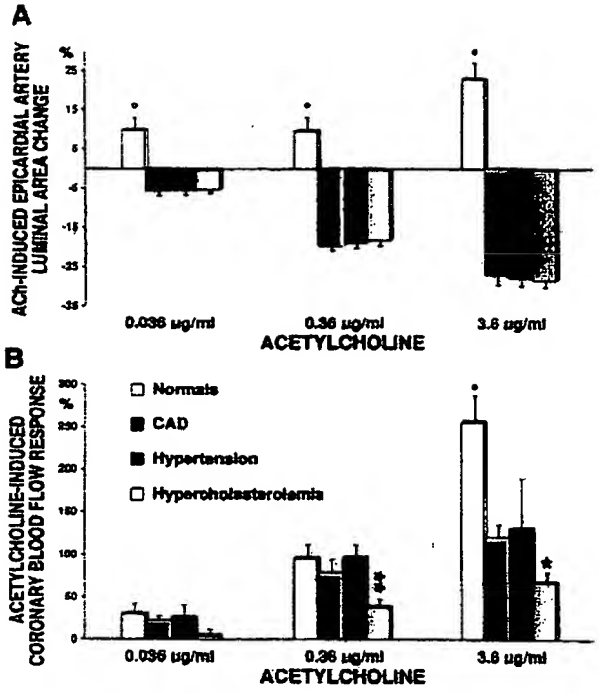

Figure 2. Dose-dependent effects of acetylcholine on epicardial artery luminal area (percentage change from baseline, *A*) and coronary blood flow (percentage change from baseline, *B*). Mean±SEM. (*Open circle*) $P < 0.01$ vs. CAD, hypertension, and hypercholesterolemia groups; (*) $P < 0.05$ vs. CAD and hypertension, and $P < 0.01$ vs. normals group; (**) $P < 0.05$ vs. normals, CAD, and hypertension group.

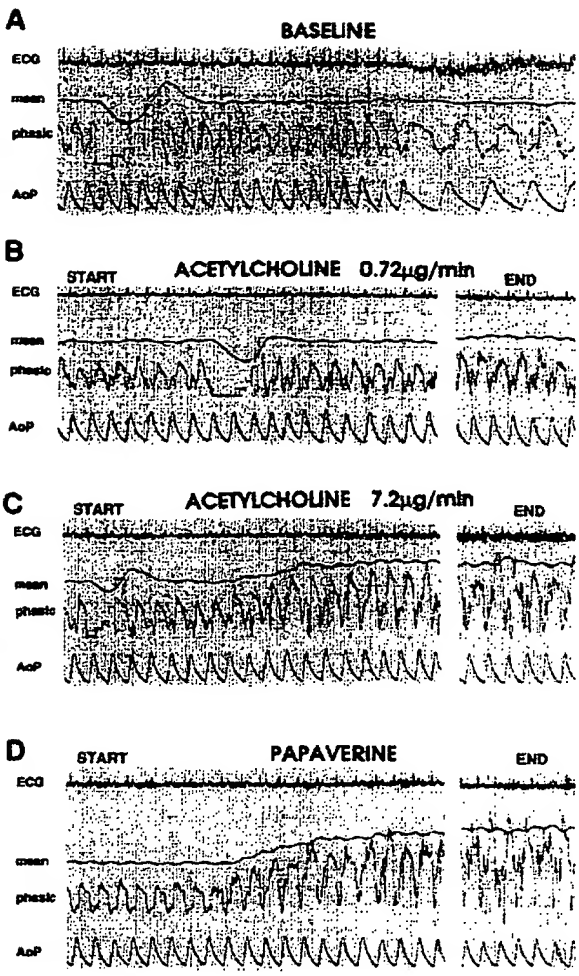

Figure 3. Coronary flow velocity tracings of the patient illustrated i Fig. 1 at baseline (*A*), at the start and end of 0.72-µg/min acetylch line infusion (*B*), at the start and end of 7.2-µg/min acetylcholine infusion (*C*), and at the start and end of papaverine infusion (*D*). Note that heart rate (*ECG*) and aortic pressure (*AoP*) are unchang during the various interventions, whereas mean blood flow velocity increases by 40% at the 0.72-µg/min acetylcholine concentration (*B* by 90% at the 7.2-µg/min acetylcholine concentration (*C*), and by 280% after 7 mg papaverine (*D*).

icance ($P < 0.05$) at the 0.36 and 3.6 µg/ml dose of acetylcholine. The dose-dependent blood flow responses to acetylcholine were similar in the normal control group, the CAD group, and the group of patients with hypertension, except for the highest dose of acetylcholine, where increases in blood flow were also significantly blunted in both the CAD group and the hypertension group compared with the normal control group (Fig. 2 *B*). The slope of the acetylcholine dose–response relation (percent change in coronary blood flow index/dosage of acetylcholine) was 81.9±39.3 in the normal control group, 43.4±13.2 in the CAD group ($P < 0.05$ vs. normal control group), 50.5±27.6 in the hypertension group ($P < 0.05$ vs. normal control group), and 24.6±14.9 in the hypercholesterolemia group ($P < 0.01$ vs. normal control group and $P < 0.05$ vs. both coronary artery disease and hypertension groups). These data demonstrate that, despite similar vasoconstrictor responses of the epicardial conductance vessels, acetylcholine-induced dilation of the coronary microvasculature is impaired in patients with hypercholesterolemia compared with patients with atherosclerosis and hypertension but normal total serum cholesterol levels. Fig. 4 illustrates that there was no relation between basal epicardial artery luminal area and acetylcholine-induced blood flow responses, indicating that intrinsic coronary artery size did not determine blood flow responses to acetylcholine.

To determine whether hypercholesterolemia affects smooth muscle function directly, the coronary blood flow r sponses to the smooth muscle relaxant papaverine were e amined. Coronary blood flow in response to papaverine i creased by 476.1±127.7% in the normal control group, 335.2±104.1% in the CAD group ($P < 0.05$ vs. normal contr group), by 336.7±105.7% in the hypertension group ($P < 0.$ vs. normal control group), and by 356.9±150.6% in the hype cholesterolemia group ($P < 0.05$ vs. normal control group Thus, although the normal control group demonstrated a sign icantly higher endothelium-independent coronary blood flo reserve, there were essentially no differences between t groups of patients with atherosclerosis, hypertension, or hype cholesterolemia.

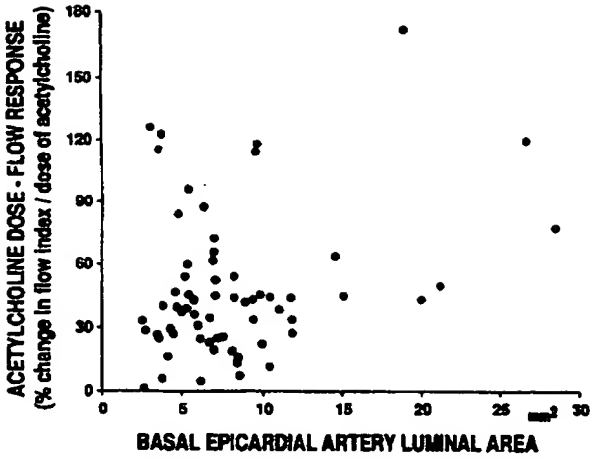

Figure 4. Relation between basal epicardial artery luminal area and acetylcholine-induced coronary blood flow changes.

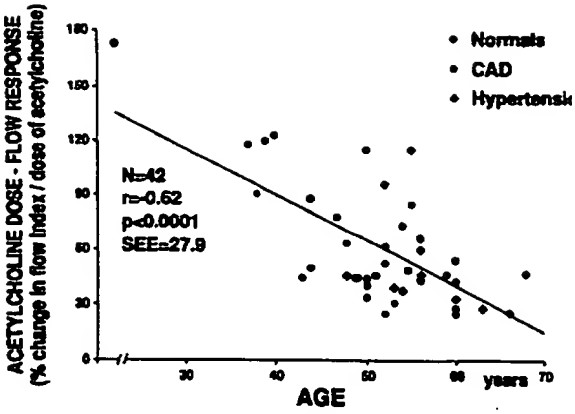

Figure 6. Correlation between age and acetylcholine dose–coronar blood flow response for all normocholesterolemic patients.

To assess the capacity of the coronary system to increase blood flow in response to acetylcholine, the acetylcholine dose–response relation was expressed as relative proportion of the maximally obtainable coronary blood flow response to papaverine. Fig. 5 illustrates that this proportion was significantly lower in patients with hypercholesterolemia compared with the three groups of patients with normal total serum cholesterol levels. When the group of patients with hypercholesterolemia was divided into those with and without an additional history of hypertension, the capacity to increase blood flow in response to acetylcholine was exactly comparable with 21.3±9.5% for the patients with hypercholesterolemia alone (n = 10) and 22.9±15.9% for the patients with both hypercholesterolemia and a history of hypertension (n = 10). Thus, the presence of a history of hypertension did not further impair acetylcholine-induced blood flow responses in patients with elevated serum cholesterol levels. Moreover, there was essentially no difference in the endothelium-dependent dilator capacity of the coronary microvasculature between the age-matched normal control group and the patients with atherosclerosis or hypertension, despite opposite vasomotor responses of the epicardial conduit vessels in these patients. These data indicate that endothelium-dependent vasodilation of the coronary microvasculature is selectively impaired in patients with hypercholesterolemia.

Relationship of acetylcholine-induced blood flow response with age and serum cholesterol levels. Multivariate analysis using stepwise multiple-regression techniques revealed a significant negative correlation of acetylcholine-induced coronary blood flow responses with total serum cholesterol level ($P < 0.0001$) and with age ($P < 0.0001$), whereas gender, arterial blood pressure at the time of the study, a history of hypertension, and the presence or absence of angiographically visible luminal epicardial artery irregularities were not independently related to the acetylcholine-induced coronary blood flow response.

Fig. 6 illustrates the significant negative relationship between the acetylcholine dose–response relation and age of the individual patients with normal serum cholesterol levels. A similar relationship was found when the effects of acetylcholine were expressed as proportion of the papaverine effects ($r = -0.58$, $P < 0.001$). Both, the epicardial artery vasomotor

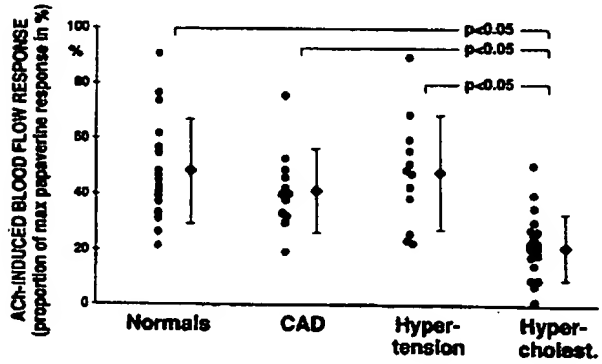

Figure 5. Capacity of the coronary system to increase blood flow in response to acetylcholine (expressed as percentage of the maximal coronary blood flow response to papaverine) of each individual patient in the four groups. Mean±SD.

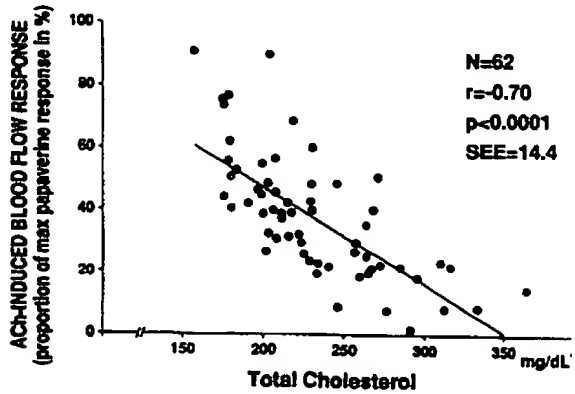

Figure 7. Correlation between total serum cholesterol level and acetylcholine-induced dilator capacity of the coronary system (expressed as percentage of the maximal papaverine response) for all patients.

response to acetylcholine as well as the papaverine-induced increases in blood flow were not significantly correlated with age ($r = 0.13$ and $r = 0.29$, $P > 0.05$, respectively). When the regression line correlating acetylcholine-induced blood flow changes and age was calculated only for the patients demonstrating a dilator response of their epicardial arteries (normal control group), the correlation coefficient improved to $r = -0.79$. Thus, the significant negative relationship between age and acetylcholine-induced blood flow responses is not solely explained by an epicardial artery vasoconstrictor response in the older patients.

The strongest single predictor of a blunted coronary blood flow response to acetylcholine was the total serum cholesterol level. Moreover, Fig. 7 demonstrates the significant negative correlation between the capacity of the coronary system to increase blood flow in response to acetylcholine and the total serum cholesterol level at the time of the study.

Discussion

The present study is the first to assess coronary blood flow responses to the endothelium-dependent agonist acetylcholine in a large series of patients with coronary atherosclerosis and different risk factors implicated in the pathogenesis of CAD. There are three important new findings. First, despite similar vasoconstrictor effects upon epicardial conductance vessels, the acetylcholine-induced increase in coronary blood flow was markedly blunted in patients with hypercholesterolemia compared with patients with atherosclerosis but normal total serum cholesterol levels. Moreover, there was a close direct relationship between total serum cholesterol and the impairment in coronary blood flow responses to acetylcholine. Second, the acetylcholine-induced dilator capacity of the coronary microvasculature decreased with advanced age irrespective of the presence or absence of epicardial artery dysfunction. Third, arterial hypertension without established left ventricular hypertrophy had no apparent effect upon acetylcholine-induced increases in coronary blood flow in the intact human coronary circulation.

Effects of acetylcholine on the human coronary vasculature

Isolated normal epicardial human coronary arteries have been shown to relax in response to acetylcholine via an endothelium-dependent mechanism, and this muscarinic endothelium-mediated relaxation is impaired in atherosclerotic arteries, leaving the direct vasoconstrictor effects of acetylcholine on the muscarinic receptors of the vascular smooth muscle unopposed (12). Clinical studies have shown that intracoronary infusion of acetylcholine dilates normal epicardial arteries in patients without risk factors for CAD (14, 16, 24), but vasoconstricts epicardial vessels in patients with risk factors for coronary atherosclerosis irrespective of the presence or absence of angiographically detectable atherosclerotic lesions (16, 27–29). The vasoconstrictor response to acetylcholine in angiographically normal epicardial arteries of patients with risk factors for CAD has been interpreted to reflect early atherosclerosis at a stage not detectable by angiography or a disturbance of endothelial function that precedes the development of atherosclerosis (16, 28). However, there is still some debate whether acetylcholine-stimulated release of EDRF also mediates vasorelaxation of the coronary resistance vasculature. This controversy stems from experimental in vitro studies, which failed to demonstrate endothelium-dependent relaxations to acetylcholine in ring preparations of microvessels (30, 31). However, when the microvascular effects of acetylcholine were investigated in pressurized arteries exposed to flow, which much more closely resembles the in vivo situation, it could be demonstrated that acetylcholine produces vascular relaxation by the release of EDRF also in the resistance vasculature of all species examined this far (21, 22, 32–34). Importantly, when methylene blue, an inhibitor of soluble guanylate cyclase, is infused into the human coronary circulation in vivo, the dilator effects of acetylcholine upon coronary microvessels are reversed to constrictor effects with dramatical increases in coronary vascular resistance (35). These results do provide indirect evidence that acetylcholine, at least in part, mediates relaxation of the human coronary resistance vasculature by the release of EDRF. In addition, when acetylcholine was infused into the brachial artery of humans, previous α-adrenoceptor blockade, as well as the administration of acetylsalicylic acid did not alter the significant reduction in forearm vascular resistance, indicating that the vascular effects of acetylcholine in the human circulation are independent of prostaglandins and adrenergic neurotransmission (36–38). However, these studies do not rule out that the endothelium-dependent dilator effect of acetylcholine is in part mediated by the release of a hyperpolarizing factor (39) nor that the dilator effect is counteracted by the concomitant release of an endothelium-derived constricting factor (33, 40). In addition, experimental studies demonstrated that flow activates an endothelial calcium-activated potassium channel to release nitric oxide (41). Moreover, although aspirin or indomethacin do not inhibit the effects of flow-stimulated endothelial cells on vascular reactivity (42), flow-induced prostacyclin release has been previously demonstrated (43). Thus, acetylcholine-induced increases in blood flow might be modulated by flow-dependent mechanisms unrelated to its effect upon endothelial muscarinic receptor stimulation. In the present study, acetylcholine increased coronary arterial blood flow by ~ 250% in those patients, who demonstrated a dilator response of their epicardial arteries to acetylcholine. This endothelium-mediated increase in coronary blood flow corresponds to roughly one half of the maximally achievable increase in blood flow by the smooth muscle relaxant papaverine. Very similar results have been recently published by Treasure et al. (26) in a comparable group of patients using a similar methodology to assess vascular reactivity of the coronary circulation in humans. Thus, the stimulated release of EDRF activity by acetylcholine can substantially modify coronary vascular resistance in the intact human coronary circulation in vivo.

Effects of atherosclerosis, hypercholesterolemia, hypertension, and age on coronary vascular reactivity

Atherosclerosis. The demonstration of opposite effects of acetylcholine upon epicardial conductance and microvessels in patients with evidence of atherosclerosis in the present study confirms previous reports from our laboratory (16) as well as by others (35, 44). Compared with the normal control patients, the blunted increase in coronary blood flow in these patients, especially at the highest dose of acetylcholine, might be explained by the constriction of the epicardial arteries, leading to a reduced vascular conductivity and thereby limiting

increases in blood flow especially during high-flow states. However, in the same subjects, the response to the endothelium-independent smooth muscle relaxant papaverine was also significantly reduced compared with the normal control group. When the coronary blood flow effects of acetylcholine were expressed as a proportion of the maximum papaverine-inducible effects, there was essentially no difference between these two age-matched groups of patients. Thus, the blunted increase in coronary blood flow in patients with atherosclerosis implicates functional abnormalities intrinsic to the vascular smooth muscle rather than the consequence of an abnormal response of either large or small coronary vessels to acetylcholine.

Hypercholesterolemia. In contrast, in patients with hypercholesterolemia, the coronary blood flow response to acetylcholine was considerably impaired in comparison to the patients with normal cholesterol levels despite similar reductions in epicardial artery cross-sectional areas and similar papaverine-induced increases in coronary blood flow. Thus, the patients with hypercholesterolemia exhibited a selective impairment of their coronary microvasculature to relax in response to acetylcholine consistent with our previous observation (16). In contrast to hypercholesterolemic patients, impaired endothelial function appears to be restricted to epicardial arteries in age-matched patients with evidence of coronary atherosclerosis but normal cholesterol levels.

Importantly, the blunted blood flow response to acetylcholine was directly related to the total serum cholesterol level. The mechanisms responsible for the blunted acetylcholine-induced relaxation of human coronary microvessels in hypercholesterolemia remain to be determined. It has been debated whether hypercholesterolemia per se or the atherosclerotic process is responsible for abnormal endothelium-dependent responses observed in experimental animals made atherosclerotic by high-cholesterol diets (22, 45). Histological specimens examined by light microscopy did not reveal any appreciable structural alterations within the vessel wall of resistance vessels obtained from cholesterol-fed animals (20, 46), although electron microscopy demonstrated the presence of vacuoles likely representing lipid droplets within the endothelium (22). It is conceivable that the accumulation of lipids with associated oxidative processes may be in large part responsible for decreased production and/or increased intracellular destruction of EDRF. Interestingly, in this context, a very recent study indicated that oxidized LDL interferes with receptor-operated signal transduction mechanisms linked to the formation of EDRF, specifically with the receptor-mediated intracellular availability of L-arginine (47). Indeed, the supplementation of L-arginine either by intravenous infusion in vivo or by in vitro exposure normalizes endothelium-dependent responses in conduit and resistance vessels of hypercholesterolemic animals without affecting endothelium-independent vascular function but has no effects in normal animals (48, 49). We have previously demonstrated that very early in the process of atherosclerosis during hypercholesterolemia in humans, endothelium-dependent responses are impaired first by a depressed receptor-mediated initiation of the production and/or release of EDRF, whereas flow-dependent dilation, which is strictly endothelium-dependent but bypasses receptor-mediated mechanisms, is well preserved (16). More importantly, we have recently demonstrated that the administration of L-arginine normalizes coronary blood flow responses to acetylcholine in vivo in hypercholesterolemic humans but has no effect on acetylcholi induced blood flow responses in patients with atherosclerc and normal levels of cholesterol (50). Thus, an interference lipoproteins with the receptor-mediated intracellular availal ity of L-arginine, the precursor of EDRF, might indeed play important role for the blunted acetylcholine-induced incre in coronary blood flow observed in patients with eleva serum cholesterol levels.

Hypertension. A history of hypertension had no appar effect upon coronary blood flow responses to acetylcholine our patients, who had no evidence of left ventricular hyp trophy as well as similar endothelium-independent blood fl responses to papaverine as the patients with atherosclerosis a the patients with hypercholesterolemia. In addition, the com nation of hypertension and hypercholesterolemia did not f ther impair acetylcholine-induced blood flow responses co pared with hypercholesterolemia alone. These results sugg that as long as hypertension has not already induced left ventu ular hypertrophy and thereby affected endothelium-indep dent coronary flow reserve to papaverine, endothelial dysfu tion of the coronary circulation in hypertensive patients is c fined to the large epicardial vessels, which are continuou exposed to high pulsatile pressure and shear stress (51, 5 These findings contrast with the results of studies in the hum forearm circulation, where arterial hypertension has b shown to be associated with a blunted blood flow response acetylcholine despite normal responses to nitroprusside (37). These discrepancies might be simply related to the dif ent vascular beds. Whereas the epicardial arteries, such as large arteries of the cerebral and limb circulation, are kno targets of hypertension (53), hypertensive vascular dise rarely develops in the large vessels of the human forearm cir lation.

Aging. Autopsy studies have shown that epicardial co nary atherosclerosis begins with fatty streaks in childhood a progresses with increasing age (54). In addition, the proxi segments of the left anterior descending artery have b shown to be particularly vulnerable to fatty streaks, as well a more advanced lesions (55). Consequently, the loss of ace choline-induced epicardial artery dilation and its reversal t vasoconstrictor response with increasing age observed in tients with angiographically normal appearing coronary ar ies have been attributed to the presence of early atherosclerc lesions in the older patients rather than to age per se (28, 2 In contrast, in the present study, the strongest relation betw age and acetylcholine-mediated dilator capacity of the co nary microvasculature was observed in those patients who hibited a dilator response of their epicardial conductance v sels to acetylcholine.

Since acetylcholine is invariably a constrictor of atheros rotic large-size arteries (12) and atherosclerosis occurs m frequently in the proximal than in the distal segments of co nary arteries (55), and does not develop at all in the coron resistance vasculature, a dilator response of epicardial cond tance vessels excludes the presence of atherosclerotic lesion least to the extent of impairing acetylcholine-mediated en thelial function. Thus, the reduction in the acetylcholine-duced dilator capacity of the coronary microvasculature w increasing age cannot solely be explained by the presence atherosclerotic lesions in the older individuals of these pati groups. These results strongly suggest that aging per se contr

utes to the impaired acetylcholine-induced dilator capacity of the human coronary microvasculature.

The mechanisms responsible for the age-associated reduction in the ability of the coronary microvasculature to dilate in response to the pharmacological stimulation with acetylcholine remain to be determined. Experimental studies demonstrating thinning and loss of endothelial cells in aged animals (56) suggested an impaired production of EDRF (57). In addition, advanced glycosylation end products, which accumulate in the vascular subendothelium in aging (58), have been experimentally shown to quench nitric oxide and mediate defective endothelium-dependent vasodilation (59). Other possible mechanisms include a decline in endothelial muscarinic receptor density, the release of hyperpolarizing or constricting factors, or an increased sensitivity of vascular smooth muscle to the constrictor effect of acetylcholine with advancing age.

Limitations of this study

The methodology used to assess coronary vascular resistance provides no direct data to identify the specific site of the impaired dilator response of the coronary microvessels in hypercholesterolemia or advanced age. Although flow-limiting vasoconstriction of epicardial conductance vessels was excluded, the precise site of coronary vascular abnormalities downstream from the epicardial vessels cannot be assessed in the intact human coronary circulation. This might be important in light of recent experimental studies demonstrating that the control of coronary vascular resistance is extremely complex and different size classes of coronary microvessels exhibit heterogeneous responses to vasoactive stimuli (60).

It has been repeatedly demonstrated that in humans, the most important vasodilator action of acetylcholine is mediated through the release of EDRF (35–38, 61). Moreover, the demonstration that the supplementation of L-arginine normalizes the blunted coronary blood flow responses to acetylcholine in hypercholesterolemic subjects (50) does provide very strong support for the existence of an acetylcholine-stimulated L-arginine/nitric oxide pathway as an important mediator of acetylcholine-induced vasodilation in the intact human coronary circulation. However, we cannot exclude the possibility that the concomitant release of a hyperpolarizing factor (39) or even a constricting factor (33, 40) or functional alterations in the flow-induced activation of endothelial potassium channels (41) might modify the response to acetylcholine during aging or hypercholesterolemia. Finally, studies in the intact human coronary circulation do not allow us to differentiate whether the impaired acetylcholine-mediated vascular relaxation is due to an abnormal production or destruction of EDRF or to abnormalities of endothelial cell membrane receptor–second messenger interactions. Although papaverine is a potent smooth muscle relaxant to assess maximal vasodilator capacity of the coronary circulation, it does not act via the same mechanism as nitrovasodilators, including EDRF. However, nitroglycerine has minimal effects on small coronary resistance vessels (62) and nitroprusside in intracoronary dosages necessary to maximize coronary blood flow in humans profoundly affects systemic hemodynamics, thereby preventing interpretation of its effects on coronary vascular resistance. Thus, we cannot exclude that aging and hypercholesterolemia might reduce the effects of acetylcholine by inactivating guanylate cyclase of vascular smooth muscle. Nevertheless, even if vascular smooth muscle guanylate cyclase is altered in hypercholesterolemia and by aging, the net effect of EDRF activity released from the endothelium upon stimulation would be a diminished relaxation of vascular smooth muscle. Thus, our conclusion of an impaired acetylcholine-induced vasodilator capacity of the coronary microvasculature with increasing age and in hypercholesterolemia with all its implications would still be valid.

Clinical significance

We have recently demonstrated that patients with endothelial dysfunction of the coronary microvasculature exhibit an impaired coronary blood flow regulation during sympathetic stimulation associated with increased myocardial work (63). Thus, the blunted endothelium-mediated vasodilator capacity of the coronary microvasculature in patients with hypercholesterolemia and advanced age might contribute to the pathogenesis of myocardial ischemia. In addition, the functional integrity of the endothelium plays a pivotal role in guarding against the initiation of vascular events that may lead to the development of vasospasm and thrombosis (1). We have recently shown that intracoronary platelet aggregation causes profound constriction of atherosclerotic epicardial arteries in humans in vivo (64). Two recent studies (65, 66) demonstrated that the intracoronary infusion of serotonin induces a dilator response in normal coronary vessels but causes profound vasoconstriction of both epicardial conductance and coronary resistance vessels in patients with arteriosclerosis, thereby suggesting an important protective effect of the endothelium. Moreover, even short-term, diet-induced hypercholesterolemia considerably increases infarct size as well as ischemia-reperfusion injury in experimental animals (21, 67, 68). In light of recent experimental evidence that the endothelium of the coronary microcirculation is extraordinarily sensitive to ischemia, responding with loss of natural anticoagulant and vasorelaxing properties (69), it is very intriguing to hypothesize that the blunted endothelium-mediated dilator capacity of the coronary microvasculature may play an important pathophysiological role for the aggravated sequelae of ischemic events in elderly and hypercholesterolemic patients with ischemic heart disease.

Acknowledgments

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Antiatherosclerotic Effects of Licorice Extract Supplementation on Hypercholesterolemic Patients: Increased Resistance of LDL to Atherogenic Modifications, Reduced Plasma Lipid Levels, and Decreased Systolic Blood Pressure

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OBJECTIVE: We previously demonstrated the beneficial effects of dietary flavonoids derived from the ethanolic extract of licorice root against atherosclerotic lesion development in association with inhibition of low-density lipoprotein (LDL) oxidation in atherosclerotic mice. Administration of licorice extract to normolipidemic subjects also inhibited LDL oxidation. In the present study, we extended our investigation to analyze the antiatherogenic effects of licorice-root extract consumption in moderately hypercholesterolemic patients.

METHODS: Supplementation of licorice root extract (0.1 g/d) to patients for 1 mo was followed by an additional 1 mo of placebo consumption.

RESULTS: Licorice consumption 1) reduced patients' plasma susceptibility to oxidation (by 19%); 2) increased resistance of plasma LDL against three major atherogenic modifications: oxidation (by 55%), aggregation (by 28%), and retention, estimated as chondroitin sulfate binding ability (by 25%); 3) reduced plasma cholesterol levels (by 5%), which was due to a 9% reduction in plasma LDL cholesterol levels; and 4) reduced (by 14%) plasma triacylglycerol levels. After the 1 mo of placebo consumption, these parameters reversed toward baseline levels. Licorice extract supplementation also reduced systolic blood pressure by 10%, which was sustained during the placebo consumption.

CONCLUSIONS: Dietary consumption of licorice-root extract by hypercholesterolemic patients may act as a moderate hypocholesterolemic nutrient and a potent antioxidant agent and, hence against cardiovascular disease. *Nutrition* 2002;18:268–273. ©Elsevier Science Inc. 2002

KEY WORDS: licorice, flavonoids, lipid peroxidation, low-density lipoprotein, blood pressure, atherosclerosis, hypercholesterolemia

INTRODUCTION

Coronary artery disease develops as a result of risk factors such as increased plasma low-density lipoprotein (LDL) level and hypertension or LDL atherogenic modifications such as retention, oxidation, and aggregation. During early atherogenesis, circulating LDLs invade the arterial wall, where it binds to extracellular matrix proteoglycans, a process known as "LDL retention".[1] LDL retention in the arterial wall may be a prerequisite to lipoprotein oxidative modification because retention of LDL to arterial proteoglycans increases its susceptibility to oxidation.[2] The process of LDL oxidation appears to occur within the artery wall, and all major artery wall cells including endothelial cells, smooth muscle cells and monocyte-derived macrophages, can oxidize LDL.[3] Extensive oxidation of LDL also leads to its aggregation,[4] and oxidized and aggregated LDL have been found in atherosclerotic lesions.[5] These LDL modifications are considered atherogenic because they contribute to macrophage cholesterol accumulation and foam cell formation, the hallmark of early atherosclerosis.

High-density lipoprotein (HDL), in contrast, is associated with antiatherogenic activity, and HDL levels are inversely related to the risk of developing atherosclerosis. Paraoxonase (PON1) is an enzyme physically associated in serum with HDL and has been shown to protect LDL and HDL against oxidation.[6]

Consumption of polyphenolic flavonoids in the diet was inversely associated with morbidity and mortality from coronary heart disease.[7] Polyphenolic flavonoids may prevent coronary artery disease by reducing plasma cholesterol levels and their ability to inhibit LDL oxidation.[8–11] The antioxidant activity of flavonoids is related to their chemical structure.[12] We previously demonstrated the beneficial effects of flavonoids from red wine,[13,14] pomegranate juice,[15] ginger extract,[16] and olive oil[17] against LDL oxidation. Further, flavonoids were shown to preserve PON1 activity[18] and increase its activity in mice[14] and humans[15] after consumption.

Licorice root derived from the plant *Glycyrrhiza glabra* is used widely in Asia as a sweetener or a spice. Licorice root contains flavonoids from the flavan and chalcone subclasses, which have lipophilic characteristics and antioxidative properties.[19] Among

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several flavonoids that were isolated and purified from licorice-root extract, the isoflavan glabridin constituted the major flavonoid.[20] We previously demonstrated that in vitro licorice extract and purified glabridin protect LDL from oxidation induced by copper ions or free radical–generating systems.[21] Mechanistic studies have shown that licorice-derived glabridin binds to the LDL particle and protects it from oxidation because of its capacity to scavenge free radicals.[21,22] Structural studies have found that the antioxidant effect of glabridin resides mainly in the 2′-hydroxyl group of the isoflavan ring B.[23] Glabridin accumulates in macrophages and inhibits cell-mediated oxidation of LDL due to the inhibition of macrophage nicotinamide adenine dinucleotide phosphate oxidase activity.[24] In vivo studies have shown that supplementation of licorice extract or glabridin to atherosclerotic mice deficient in apolipoprotein E (E^0) reduces the susceptibility of their LDL to oxidation and significantly reduces the development of aortic atherosclerotic lesions.[21,24] In another study, licorice extract was administered to healthy normolipidemic subjects for 2 wk; consumption of 100 mg/d of licorice root extract increased their LDL resistance against oxidation.[21]

In the present study we extended our investigation on the antiatherogenic properties of licorice-root extract in moderately hypercholesterolemic patients. We found that licorice-root extract consumption by mildly hypercholesterolemic patients increases the resistance of their LDL against major atherogenic modifications and moderately reduces their plasma lipid levels and systolic blood pressures.

METHODS AND MATERIALS

Materials

Licorice ethanolic extract free of glycyrrhizinic acid was provided by Fertilizers & Chemicals Ltd. (Haifa, Israel). Powdered roots of commercial *Glycyrrhiza glabra* were extracted in ethanol to obtain, after solvent evaporation, a brown solid extract. The powder was encapsulated in a soft-gel capsule (RP Scherer, Miami, FL, USA). Placebo capsules without licorice contained innertic gelatinous material normally included in soft-gel capsules. Na_2 ethylene-diaminetetraacetic acid (EDTA) was purchased from Sigma (St., Louis, MO, USA). 2,2′-Azobis 2-amidino propane hydrochloride (AAPH) was purchased from Wako Chemical Industries Ltd (Osaka, Japan).

Patients

Twelve hypercholesterolemic patients (45 to 55 y old, plasma cholesterol level of 220 to 260 mg/dL, and LDL cholesterol level of 120 to 170 mg/dL) were selected for this study. All patients were non-smokers, with body weights of 78 ± 9 kg (mean ± standard deviation); none exhibited evidence of liver, kidney, endocrine, or heart disease, and none had ever been treated with hypolipidemic drugs. All patients were first administered licorice extract (0.1 g/d) in soft-gel capsules for 1 mo followed by additional month of placebo supplementation. To eliminate possible analytical drift or any other potentially confounding results, the patients and the laboratory technicians did not know which capsule (the licorice or the placebo) was consumed for the first month because both capsule types appeared the same. Therefore, this study was considered blind for all participants. The subjects' mean (± standard deviation) body mass index (kg/m^2) was 25 (±1.7) and did not change significantly during the study. All patients continued their habitual diets during the study. The consumption of the licorice extract or placebo did not alter other food intakes or diet preferences, and patients did not report any digestive or other disturbances. Blood samples were drawn after 12 h of fasting, before study entry, 1 mo after licorice administration, and 1 mo after placebo supplementation.

The study was approved by the Helsinki Committee of the Rambam Medical Center, Israel Ministry of Health (no. 1175).

Blood Pressure Measurement

Blood pressure was measured by a sphygmomanometer with random zero values. The same apparatus was used for the entire study. The measurements were made always on patients' left arms; under similar conditions. The patients were seated, and each arm was placed on a table approximately at the level of the heart. Blood pressure was measured after patients had relaxed, at two time points for each measurement, before and 30 min after blood withdrawal. The measurements did not differ significantly, and the mean value was used.

Serum Paraoxonase Activity

Serum PON1 activity was measured as arylesterase with phenylacetate as the substrate. Initial rates of hydrolysis were determined spectrophotometrically at 270 nm. The assay mixture included 1.0 mM phenylacetate and 0.9 mM $CaCl_2$ in 20 mM Tris HCl, pH 8.0. Non-enzymatic hydrolysis of phenylacetate was subtracted from the total rate of hydrolysis. The E_{270} (extinction coefficient) for the reaction was 1,310 Mcm. One unit of arylesterase activity is equal to 1 $\mu M \cdot min^{-1} \cdot mL^{-1}$ of hydrolyzed phenylacetate.

Plasma Lipid Peroxidation

Plasma was incubated with or without 100 mM/L of the free radical generator AAPH for 2 h at 37°C. Plasma lipid peroxidation was determined by measuring the generated amount of thiobarbituric acid–reactive substances (TBARS).[25]

LDL Isolation

LDL was separated from plasma by discontinuous density-gradient ultracentrifugation[26] and dialyzed against saline with EDTA (1 mM/L). Before the oxidation study, LDL was diluted in phosphate-buffered saline (PBS) to 1 g of protein/L and dialyzed overnight against PBS at 4°C to remove the EDTA. LDL protein concentration was determined with the Folin phenol reagent.[27]

LDL Oxidation

LDL (100 mg of protein/L) was incubated with 5 μM/L of $CuSO_4$ for 3 h at 37°C. The formation of conjugated dienes was continuously monitored by measuring the increase in absorbance at 234 nm.[28] Incubations were carried out in the spectrophotometer cuvette (Ultraspec 3000; Pharmacia, LKB, Biochrom Ltd., Cambridge, UK). The initial background of the samples ranged between 0.1 and 0.2 optical density (OD) as recorded at 234 nm. After initial absorbance was recorded, the spectrophotometer was set to zero against blank, and the increase in absorbance of the sample that consisted of LDL and $CuSO_4$ was recorded every 10 min during LDL oxidation. The lag time required for the initiation of lipoprotein oxidation was calculated from the oxidation curve.

LDL Aggregation

LDL (100 mg of protein/L) was mixed by vortex at a fixed strength, and the absorbance at 680 nm was monitored every 10 s against a blank solution. Results after 60 min of vortexing are reported.

LDL Retention (Chondroitin Sulfate Binding Ability)

LDL retention was estimated as the ability of LDL to bind to chondroitin sulfate (CS). LDL (200 mg of lipoprotein protein/L) was incubated with CS (100 mg/L) for 30 min at room temperature. The lipoprotein was precipitated with a commercial HDL cholesterol reagent (phosphotungstic acid and $MgCl_2$, Sigma) that precipitated all the LDL in the samples, followed by a 10-min centrifugation at 2000*g*. After discarding the supernatant, the LDL in the precipitate was dissolved in 0.1 N NaOH and analyzed for its glycosaminoglycan (GAG) content using the 1,9-dimethylmethylene blue (DMMB) spectrophotometric assay for sulfated GAGs.[29] Briefly, 2.5 mL of ice-cold DMMB working solution (46 μM/L of DMMB, 40 mM/L of glycine, 40 mM/L of NaCl in 5% ethanol, adjusted to pH 3.0) was added to 500 μL of the dissolved precipitate. The absorbance at 525 nm was immediately measured. CS was used as the standard and included within each series of assays. Similar preparation of LDL, with no CS added, was used in parallel as a control. GAG content in the control was subtracted from the GAG content in LDL preparations that were incubated with CS.

Statistics

Student's paired *t* test (one tail) compared the two arrays of data, and analysis of variance was used when more than two groups were compared. For statistical analysis of parameters without Gaussian distributions such as triacylglycerols and LDL oxidizability, the values were transformed to logarithm values to create a Gaussian distribution. Results are given as mean ± standard error of the mean.

RESULTS

After 1 mo of licorice-root ethanolic extract consumption, blood chemistry analyses showed no significant changes in markers for liver, kidney, and heart functions as measured by serum blood urea nitrogen, creatinine, alanine aminotransferase, aspartate aminotransferase, bilirubin, and creatine phosphokinase (Table I). Placebo treatment for 1 mo did not significantly affect those markers (Table I). In addition, serum electrolytes, including kalium and natrium, and serum alkaline phosphatase were not significantly affected by licorice consumption or placebo (Table I). However, a small (7%) but significant ($P < 0.01$) reduction in serum glucose and a 10% reduction in serum amylase concentrations were observed after licorice consumption (Table I). After the 1-mo placebo treatment, serum glucose and amylase levels returned to baseline values (Table I).

We next analyzed the effect of licorice-extract consumption on serum lipid profiles in the hypercholesterolemic patients (Table II). Serum cholesterol levels were minimally (5%) but significantly ($P < 0.01$) reduced after licorice consumption as a result of a 9% reduction in LDL cholesterol. Those effects were not sustained beyond the treatment period; after 1 mo of placebo treatment, serum and LDL cholesterol levels returned to baseline levels (Table II). Serum HDL cholesterol levels did not change during licorice consumption.

Serum triacylglycerol concentrations decreased by 13% after licorice consumption, as reflected by a significant ($P < 0.01$) reduction of 14% in serum VLDL levels (Table II). After the placebo treatment, serum triacylglycerol levels returned toward baseline values (Table II).

Blood pressure measurements showed a significant ($P < 0.01$) 10% reduction in systolic blood pressure, with no significant effect on the diastolic blood pressure (Fig. 1). After the 1-mo placebo treatment, the decrease in systolic blood pressure remained compared with baseline levels (Fig. 1).

Analysis of the effect of licorice consumption on oxidative stress, a major risk factor for cardiovascular diseases, was performed in serum samples and LDL fractions isolated from the patients' serum before and after licorice consumption and after placebo consumption.

Serum PON1, measured as arylesterase activity, was not affected by licorice consumption (215 ± 25 and 203 ± 23 U/mL of arylesterase before and after licorice consumption, respectively).

TABLE I.

BIOCHEMICAL PARAMETERS OF HYPERCHOLESTEROLEMIC PATIENTS BEFORE AND AFTER 1 MO OF LICORICE EXTRACT SUPPLEMENTATION AND AFTER AN ADDITIONAL MONTH OF PLACEBO SUPPLEMENTATION*

	Time after licorice extract supplementation		
Serum concentration	Baseline	1 mo after licorice consumption	1 mo after placebo
Glucose (mg/dL)	90 ± 3.0	83 ± 1.0†	88 ± 1.0
Blood urea nitrogen (mg/dL)	14 ± 0.7	13 ± 0.5	14 ± 0.6
Creatinine (mg/dL)	0.9 ± 0.03	0.9 ± 0.04	0.9 ± 0.04
Kalium (mEq/L)	4 ± 0.1	5 ± 0.1	5 ± 0.1
Natrium (mEq/L)	140 ± 1.0	140 ± 1.0	140 ± 1.0
Calcium (mg/dL)	9 ± 0.1	9 ± 0.3	10 ± 0.2
Amylase (U/L)	82 ± 8.0	74 ± 8.0†	81 ± 7.0
Aspartate amino transferase (U/L)	22 ± 1.0	23 ± 1.0	24 ± 1.0
Alanine amino transferase (U/L)	23 ± 3.0	22 ± 2.0	23 ± 2.0
Alkaline phosphatase (U/L)	69 ± 5.0	73 ± 5.0	75 ± 5.0
Creatine phosphokinase (U/L)	86 ± 7.0	91 ± 13.0	93 ± 12.0
Bilirubin, total (mg/dL)	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1

* Results are expressed as mean ± standard error of the mean (each assay was performed in duplicates).
† $P < 0.001$, versus baseline at study entry.

TABLE II.

EFFECT OF LICORICE EXTRACT SUPPLEMENTATION ON HYPERCHOLESTEROLEMIC PATIENTS ON THEIR PLASMA LIPIDS AND LIPOPROTEINS*

	Time after licorice extract supplementation		
Serum concentration (mg/dL)	Baseline	1 mo after licorice consumption	1 mo after placebo
Cholesterol	244 ± 8	232 ± 9†	252 ± 7
LDL cholesterol	156 ± 8	142 ± 9†	158 ± 8
HDL cholesterol	54 ± 2	54 ± 5	55 ± 5
VLDL cholesterol	36 ± 5	31 ± 4†	36 ± 5
Triacylglcerols	174 ± 23	155 ± 18	184 ± 23

* Results are expressed as mean ± standard error of the mean (each assay was performed in duplicates).
† $P < 0.01$, after 1 mo of licorice consumption versus baseline at study entry.
HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein

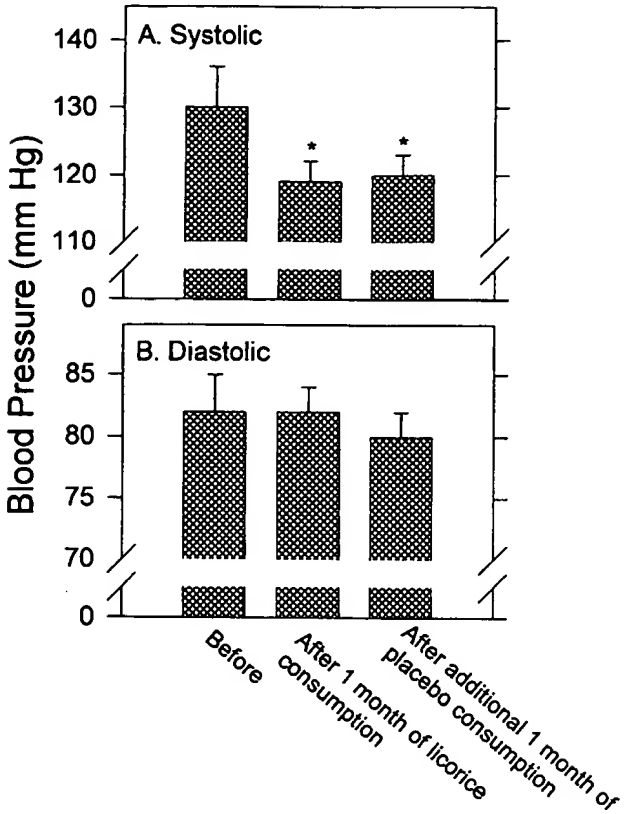

FIG. 1. The effect of licorice extract supplementation on on systolic (A) and diastolic (B) blood pressures of hypercholesterolemic patients. Blood pressure was measured before licorice consumption, 1 mo after licorice consumption, and after an additional 1 mo of placebo consumption. Results are expressed as mean ± standard error of the mean. $^*P < 0.01$, versus before licorice consumption.

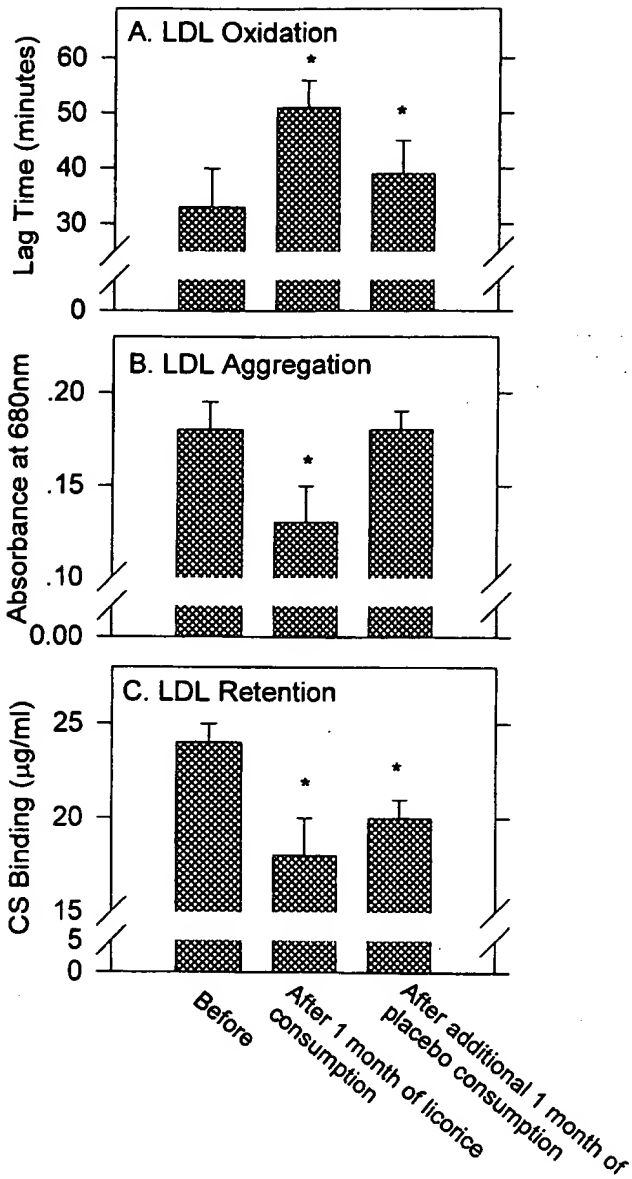

FIG. 2. The effect of licorice extract supplementation on the susceptibility of LDL in hypercholesterolemic patients to atherogenic modifications: oxidation (A), aggregation (B), and binding ability (C). LDL was isolated from hypercholesterolemic patients before, 1 mo after licorice extract supplementation, and an additional 1 mo after placebo supplementation. (A) LDL (100 mg of protein/L) was incubated with 5 µmol/L of $CuSO_4$ for 3 h at 25°C. The formation of conjugated dienes was kinetically monitored at 234 nm and the lag time was measured. (B) The extent of LDL aggregation induced by vortexing was kinetically monitored at 680 nm, and results after 60 s of vortexing are shown. (C) LDL (200 mg of protein/L) was incubated with CS (100 mg/L) for 30 min at 37°C. LDL was then precipitated, and the LDL-associated glycosaminoglycan content in the precipitate was measured. Results are expressed as mean ± standard error of the mean. $^*P < 0.01$ versus baseline at study entry. CS, chondroitin sulfate; LDL, low-density lipoprotein.

Plasma after 1 mo of licorice extract consumption showed a significant ($P < 0.01$) 19% decreased susceptibility to the free radical generator AAPH-induced lipid peroxidation, measured as TBARS formation, in comparison with plasma obtained before licorice extract supplementation (37±1 mM before versus 30 ± 1 mM/L after licorice consumption, TBARS/L of plasma). This effect was not sustained after the 1 mo of placebo-treatment, and the AAPH-induced serum lipid peroxidation returned toward baseline values (34 ± 2 mM of TBARS/L of plasma).

We next analyzed the effect of licorice extract supplementation on the resistance of hypercholesterolemic patients' LDL to three major atherogenic modifications: LDL oxidation, LDL aggregation, and CS binding ability. The susceptibility of LDL from hypercholesterolemic patients after consumption of licorice extract for 1 mo to copper ion-induced oxidation was reduced, as shown by the prolonged lag time of 55% required for the initiation of LDL oxidation in comparison with the lag time of LDL isolated from plasma derived before licorice extract consumption (Fig 1A). This effect was partly sustained after 1 mo of placebo supplementation because the LDL derived after that period was still less susceptible to copper ion-induced lipid peroxidation, as demonstrated by an 18% increment in the lag time, in comparison with the baseline lag time (before licorice administration, Fig. 2A).

The atherogenicity of LDL has been attributed to its oxidative modification and its aggregation.[2-5] LDL oxidation lead to its subsequent aggregation,[4,30] and we recently reported that polyphenols can reduce LDL aggregation in vitro and ex vivo.[10,11] After analyzing the susceptibility to aggregation of LDL isolated from hypercholesterolemic patients who had consumed licorice extract for 1 mo, we found a significant ($P < 0.01$) reduction of 28% in LDL aggregation (Fig. 2B). After 1 mo of placebo consumption, LDL aggregation rates returned toward baseline values.

Retention of LDL, which is an early step in atherogenesis, was measured by analysis of LDL binding to the proteoglycan CS.

After licorice consumption, LDL CS binding ability decreased significantly ($P < 0.01$) by 25% and that effect was partly sustained during the 1 mo of placebo consumption (Fig. 2C).

DISCUSSION

Consumption of licorice-root extract was shown to inhibit oxidative modification of LDL in healthy human volunteers and accelerated development of atherosclerotic lesions in E^0 mice.[21] The present study expanded those findings to hypercholesterolemic patients and showed that consumption of licorice extract increases the resistance of LDL to atherogenic modifications, including CS binding ability, oxidation, and aggregation, and moderately reduced their plasma lipids levels and systolic blood pressures. The lipid peroxidation hypothesis of atherosclerosis is supported by the antiatherosclerotic effects of some nutritional antioxidants, as demonstrated in humans and E^0 mice.[2,3,7–10] Licorice extract exhibited antioxidative characteristics against LDL oxidation in normal healthy subjects.[21] We previously showed that LDL from hypercholesterolemic patients is highly-susceptible to oxidation.[31] In the present study, licorice extract consumption by hypercholesterolemic patients significantly reduced the increased susceptibility of their LDL to oxidation, further demonstrating its remarkable antioxidative capacity ex vivo. The mechanisms responsible for licorice protection of LDL against oxidation lie in its previously shown capacity to bind to LDL, scavenge free radicals, and protect other antioxidants associated with LDL, the carotenoids, from oxidation.[21–23] However, licorice consumption did not affect two other protectors of LDL from oxidation in serum; plasma HDL cholesterol level and serum PON1 activity. Thus, our results suggest that the protection of LDL against oxidation by licorice consumption is not mediated by an increase in HDL or PON1 activity but by a direct effect on LDL itself. These results are in contrast to what we previously found with dietary supplementation of other polyphenolic antioxidants-rich nutrients[14,15,18] such as red wine and pomegranate juice, which increased serum PON1 activity.

Atherosclerosis is a multifactorial disease, and other factors besides lipid peroxidation can accelerate atherogenesis independently or in association with lipid peroxidation. Increased retention and aggregation of LDL in the arterial wall and LDL oxidation are key events in the acceleration of atherogenesis. The present study showed for the first time that licorice extract consumption by hypercholesterolemic patients reduces the susceptibility of their LDL to ex vivo CS binding ability, oxidation, and aggregation.

We used vortexing to assess the susceptibility of LDL to aggregation. This method was described previously as a physical procedure that leads to the exposure of the lipoprotein surface hydrophobic domains, leading to surface-induced aggregation of LDL.[32] Because LDL is sensitive to surface denaturation, this method, although quite aggressive, might represent changes that apparently occur during the retention of LDL in the arterial wall. There are several lines of evidence that LDL aggregation occurs in the arterial wall.[5,33] Further, we recently demonstrated that macrophage-released proteoglycans are involved in macrophage stimulation of LDL aggregation.[34] Because LDL and macrophages are retained in the atherosclerotic lesion on the arterial wall and macrophages release proteoglycans to their surroundings under atherogenic conditions, the macrophage-mediated aggregation of LDL may play a physiologic role in LDL modification in vivo. Further, because aggregated LDL can induce macrophage cholesterol accumulation and foam cell formation[30] it is considered as a risk factor for atherosclerosis. The inhibitory effect of licorice on LDL aggregation can be attributed to possible binding of licorice extract constituents such as its polyphenols to the LDL particle, and such interactions between the lipoprotein hydrophobic domains and the licorice polyphenols can affect interactions between lipoproteins and their subsequent aggregation. These results are in agreement with those of our previous study, which demonstrated a reduced susceptibility to aggregation of LDL derived from atherosclerotic E^0 mice after dietary consumption of red wine or its major polyphenols catechin or quercetin.[14]

The reductions in the susceptibility of LDL to oxidation, its CS binding ability, and blood pressure after licorice consumption were partly sustained during the month of placebo consumption. These effects could be related to the persistance of some licorice-derived components in plasma and its association with newly formed LDL, thus further protecting it from modification for an extended period over the supplementation period. However, the susceptibility to aggregation was not sustained over the supplementation period, suggesting that additional mechanisms beyond the interrelations of oxidation and aggregation are involved in licorice-mediated inhibition of LDL aggregation.

Administration of licorice extract to hypercholesterolemic patients significantly reduced LDL oxidation and aggregation and moderately reduced their plasma and LDL cholesterol levels. This hypocholesterolemic effect of licorice extract is in agreement with a previous report showing that plant foods possess cholesterol-suppressive capacities, in agreement with our previous report that the antioxidant carotenoids β-carotene and lycopene in tomatoes act as hypocholesterolemic agents, secondary to their inhibitory effect on cellular cholesterol biosynthesis.[35]

In addition to the inhibitory effect of licorice consumption on LDL quality (i.e., susceptibility to oxidation) and quantity (serum concentration), licorice extract consumption induced a moderate reduction in patients' systolic blood pressures. The licorice ethanolic extract used in our study is completely free of glycirhizinic acid, a known hypertensive agent. The hypotensive effect of dietary antioxidants has been reported in hypertensive patients.[36] Because reactive oxygen species contribute to endothelium-dependent contraction and increase vascular resistance, antioxidants might restore endothelial function and, hence, decrease blood pressure.

We conclude that consumption of licorice extract may prove beneficial in its ability to attenuate the accelerated development of atherosclerosis in hypercholesterolemic patients. Licorice-root extract consumption was associated with reduced atherogenic modifications of LDL, including reduced oxidation, reduced CS binding ability (retention) and aggregation, reduced plasma lipid levels, and reduced systolic blood pressure.

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